The use of visual marker genes for investigation of regeneration pathway and development of transformation system for wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.)

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Abstract

Recent advancements in molecular biology, genetic modification technology and genomics platforms will greatly enhance plant breeding practices for meeting global demand for food, feed, fibre, fuel and even biological drugs. Development of such plant biotechnology will transform modern agriculture into sustainable, low input, high and diverse output businesses. Although the most important cereal crops, including wheat and barley, have proven to be transformable, the existing transformation methods are not yet efficient to meet the needs for research and product development. This thesis describes effort to contribute to wheat and barley transformation method development and optimisation by: (1) investing plant regeneration pathways; (2) exploiting new visual marker genes as early reporters for screening transformed cells and plants; (3) testing *Agrobacterium*-mediated wheat transformation as an alternative DNA delivery method to the current method.

Understanding of plant regeneration pathway is an intriguing scientific topic in its own right. It is also a pre-request for developing an efficient transformation method. The wheat regeneration pathway was studied after introducing foreign genes into cultured immature zygotic embryos of wheat (*Triticum aestivum* L.) by microprojectile bombardment. Three reporting systems, *gusA*, *C1/Lc* and *gfp*, were used. The results suggested clearly that regenerated wheat plants originated from single cell sources of scutellar tissue *via* a typical somatic embryogenesis.

New vital visual markers allows for refinement of transformation permutations and possibly for replacing negative selectable markers. A synthetic green fluorescent protein gene, *sgfp (S65T),* was used during studies of wheat transformation. Another visual marker, the firefly luciferase gene, *luc*, was used during studies of barley (*Hordeum vulgare* L.) transformation, together with a selectable marker, the *bar* gene, and a target, the *dapA* gene. The expression of the *sgfp (S65T)* gene in regenerating cultures was followed using epifluorescence microscopy. Green fluorescence was detected within multicellular structures as early as 12 days after microprojectile bombardment. The sequential epifluorescent screens allowed detection of the GFP signal within early embryogenic structures without the aid of a selectable marker; thus, this gene can be used as an independent co-transformation marker.

The visual marker *C1/Lc* genes were also used to study if wheat scutellum-derived callus is susceptible to *Agrobacterium*-mediated transformation. The result of red pigmentation in the target cells, as a consequence of expressing the *C1/Lc* genes transiently following the inoculation, clearly suggests that the wheat cells are transformable by *Agrobacterium*.

From this study, we would like to suggest that: 1) single cell origin of somatic embryogenesis in wheat means that DNA delivery at the right target tissue and at the right time is the key to obtain non-chimeric transgenic plants; 2) visual markers are not only tools for optimising transformation conditions, but also can be used as screening markers for wheat and barley transformation when the DNA delivery is efficient; 3) wheat cells are clearly susceptible to *Agrobacterium*-mediated DNA delivery, from the study of 1), it is important to develop conditions that allow for *Agrobacterium* to target those single progenitor cells efficiently.

Abbreviations

Ac/Ds	maize transposons *Activator/Dissociation*
AS	acetosyringone
bar	phosphinothricin acetyltransferase gene
bp	base pairs
CAT	chloramphenicol acetyltransferase
C1	maize regulatory gene involved in anthocyanin-biosynthesis
CTAB	cetyltrimethylammonium bromide
2,4-D	2,4-dichlorophenoxyacetic acid
dapA	dihydrodipicolinate synthase (DHPS) gene, a regulatory gene involved in lysine biosynthesis
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
GFP	green fluorescent protein
gfp	green fluorescent protein gene
GUS	β-glucuronidase
gusA	β-glucuronidase gene
HMW	high molecular weight
HMW-GS	high-molecular-weight glutenin subunit
hpt	hygromycin phosphotransferase gene
IE	immature embryo
kb	kilo base pairs
KT	kinetin
Lc	maize regulatory gene involved in anthocyanin-biosynthesis
LUC	luciferase
luc	luciferase gene
manA	gene encoding phosphomannose isomerase from *Escherichia coli*
MS	Murashige and Skoog basic medium
NAA	naphthaleneacetic acid
nptII	neomycin phosphotransferase II gene
OH-AS	hydroxyacetosyringone
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PEG	polyethylene glycol
pmi	phosphomannose isomerase gene
PPT	phosphinothricin
PSC	potato suspension culture
PVPP	polyvinyl polypyrrolidone
SDS	sodium dodecyl sulphate
SE	somatic embryo
SSC	0.15M sodium chloride, 0.015M sodium citrate
TAE	Tris-acetate buffer
TE	Tris-EDTA buffer
X-GLUC	5-bromo-4-chloro-3-indolyl glucuronide

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Chapter One

INTRODUCTION

1.1 Wheat and barley – the economic importance

Wheat and barley are major cereal crops which are of great economic importance as food and feed for the world. According to the Food and Agriculture Organization of the United Nations (FAO), in 2004, world cereal production was 2,252 million tonnes, 27.7% (624 million tonnes) was from wheat and 6.9% (155 million tonnes) was from barley (FAO 2005) (see **Table 1-1** for more data). Wheat and barley products take up a substantial part of the world agriculture and the food trade market. In 2003, the world wheat flour exports trade was 8.7 million tonnes with trading values of 2,006 million US dollars, and beer of barley exports trade was 8.3 million tonnes, with trading values of 6,696 million US dollars (FAO 2005) (see **Table 1-2** for more data).

Despite continuous growth of the world economy and availability of food for export by the industrial nations, the food security situation of the developing world has shown little improvement in recent years (FAO 2001). Several countries continue to face food shortages, and some 60 million people in 36 countries are facing food emergencies (FAO, 2001). According to The millennium development goals report 2005 (UN Report 2005, link http://www.fao.org/es/ess/faostat/foodsecurity/index_en.htm), there were 815 million hungry people in the developing world in 2002 — 9 million less than in 1990. Yet in the worst-affected regions — sub-Saharan Africa and Southern Asia — the number of hungry people has increased by tens of millions. Growing populations and poor agricultural productivity have been the main reasons for food shortages in these regions. For young children, the lack of food can be perilous since it retards their physical and mental development and threatens their very survival. More than a quarter of children under age 5 in developing countries are malnourished (UN Report 2005).

Table 1-1 World production of wheat, barley, and total cereal

World Production (million tonnes)	Year					
	1970	**1980**	**1990**	**1995**	**2000**	**2004**
Cereals, Total	1,193	1,550	1,952	1,897	2,060	2,252
Wheat	311	440	592	543	585	624
Barley	119	157	178	141	133	155

Note:

Data source from © FAO 2005.

At FAO homepage www.fao.org click Statistical Databases, and then click FAOSTAT-Agriculture, and then Crops Primary. Or search the Agricultural Production by go to the link:

http://faostat.fao.org/faostat/form?collection=Production.Crops.Primary&Domain=Production&servlet=1&hasbulk=&version=ext&language=EN

Table 1-2 World trade in wheat and barley products: flour of wheat and beer of barley

World Exports Trade		**Year**				
		1980	**1990**	**1995**	**2000**	**2003**
Flour of Wheat	Quantity (million tonnes)	6.8	6.9	10.5	9.0	8.7
	Value (million $)	1,930	1,763	2,735	1,780	2,006
Beer of Barley	Quantity (million tonnes)	2.2	3.8	6.4	6.2	8.3
	Value (million $)	1,136	2,714	4,734	4,812	6,696

Note:

Data source from © FAO 2005.

At FAO homepage www.fao.org click Statistical Databases, and then click FAOSTAT-Agriculture, and then Crops & Livestock Primary & Processed. Or search the Agriculture & Food Trade by go to the link:

http://faostat.fao.org/faostat/form?collection=Trade.CropsLivestockProducts&Domain=Trade&servlet=1&hasbulk=&version=ext&language=EN

Production of new wheat and barley varieties with higher yields and better grain quality is necessary to meet the world's increasing demand for food and feed products. There are two main factors affecting overall yield increases: the potential of individual varieties to deliver higher yields and the resistance characteristics that reduce the losses caused by unfavourable factors, such as drought, pests, diseases and weeds (Day and Lupton, 1987). The increases of wheat yields in the last decades can be attributed to better husbandry, and in particular to more-efficient control of pests, diseases and weeds, but it has been estimated that at least half of the increase is due to the introduction of new varieties (Day and Lupton, 1987).

Wheat is processed into many different manufactured products, such as leavened bread, cakes, biscuits and breakfast cereals, many of which require different characteristics of the grains. Barley is the most important raw material for brewing industry. Wheat and barley are also important component of many animal feed stocks. Different types of wheat and barley grain qualities are desired to meet the needs of different use purposes (Blackman and Payne, 1987). For example, wheat flour quality can be improved by introducing the genes for novel proteins, such as glutenin from wild species of *Triticum* and *Aegilops*, into wheat (Gale and Miller, 1987).

1. 2 Cereal breeding and improvement

The overall aim of cereal breeding is to produce higher yielding varieties with better quality, allied to durable disease and pest resistance, better environmental adaptation, and greater stress tolerance. Most conventional breeding programmes are based on variations of the pedigree system of selection (Day and Lupton, 1987). More recently, transgenic technology has been developed, adding a new tool for crop improvement.

1.2.1 Conventional breeding

Conventionally, improved varieties of wheat and barley have been obtained by crossing parent plants, each of which has some of the desired traits, and selecting from the progeny those individuals which show the desired combination of traits. Many breeding techniques, such as intraspecific and entrageneric crossing back-crossing, self-pollination, embryo rescuer, radiation breeding, chromosome doubling etc., have been developed (Goodman *et al* 1987). Furthermore, novel breeding lines have been established such as monosomic and additional lines in wheat and barley (Law *et al* 1987), male sterility systems in maize (Kaul 1988) and photosensitive sterility systems in rice (Shi and Deng 1986). All of these achievements have allowed breeders and plant scientists to understand better about the genetics of trait inheritance, chromosome organisation and the location of important gene alleles on chromosome in crop plants (Flavell *et al* 1987).

Over the past seven decades, conventional breeding has produced a vast number of varieties and hybrids that have contributed immensely to higher grain yield, stability of harvests, and farm income. There have been important improvements in resistance to diseases and insects, and in tolerance to a range of abiotic stresses, especially soil toxicity. World wheat grain yields increased substantially in the 1960s and 1970s (refer **Table 1-1)** because of the introduction of semi-dwarf varieties and farmers' rapidly adoption of new cultivation methods. This period of success has been widely held as "Green Revolution" (Conway, 1997). However, there are limitations resulting from the inbreeding method. Genetic erosion, which involves the gradual narrowing of genetic diversity in inbred in cultivated varieties, seriously limits further improvement of yield and quality (Gale and Miller, 1987). To overcome this problem, wild relatives of cultivated wheat and barley are identified as important sources of genetic variation for wheat and barley breeding programmes.

For wheat improvement, the successful transfer of useful genes from alien species requires that the target genes or a segment of chromosome carrying them can be incorporated into the wheat chromosomes by recombination or translocation (for review see Jiang *et al.*, 1994). The general procedure to achieve this goal is to produce, first, F_1 hybrids between wheat and alien species, then stable amphiploids followed by wheat-alien chromosome addition and substitution lines, and finally wheat-alien chromosome translocations, which are induced by exploiting homologous chromosome pairing (Riley *et al.*, 1968; Sears, 1981), ionizing irradiation (Sears, 1956), or tissue culture (Lapitan *et al.*, 1984). An alternative approach, using anther culture techniques, has been proposed (Hu *et al.*, 1988). Alien addition and substitution lines, sometimes even addition lines can be obtained directly by culturing the anthers of wheat/alien hybrids (Wang and Hu, 1985; Tao and Hu, 1989; Wang *et al.*, 1996).

In spite of the impressive achievements, cereal improvement by crossbreeding has its limitations. Only traits present in related species can be combined. Crosses between distantly or unrelated species yield sterile plants or no progeny at all. Furthermore, during crossbreeding all the traits, good and bad, of both partners are recombined. These results in progeny which have the desired traits combined with a number of unwanted ones. Thus, a time consuming backcrossing programme, in most cases eight to twelve generations, is necessary to eliminate the unwanted traits. Moreover it is sometimes not possible to completely remove the unwanted traits (BIOTOL 1991).

1.2.2 Crop biotechnology

Conventional breeding continues to be an essential method for crop improvement. Biotechnology has emerged during recent decades to provide an additional approach to plant improvement (BIOTOL 1991). Tissue culture techniques combined with recombinant DNA technology make it possible to add isolated genes for desired traits into

existing élite plant varieties. Because these techniques are not based on the usual sexual process in which all genes are recombined, time spent on backcrossing can be reduced (BIOTOL 1991). Thus the breeding procedure is accelerated. Also, the pool for candidate genes is broadened, genes from unrelated species/organism may be used, though with care and after necessary modification, for improvement of crop plants.

In the early 1980s, the first transgenic plants expressing foreign genes were successfully produced (Horsch *et al.*, 1984; De Block *et al.*, 1984). This marked the start of exploitation of biotechnology for crop enhancement and protection. Since then, a wide range of species, including many agronomically important crops, have been transformed. Transgenic plants with desirable resistance properties and quality traits have been produced (see Dale *et al.*, 1993 for a review). Biotechnology has great potential for improving wheat quality (Shewry *et al.*, 1995). Genes encoding novel high molecular weight (HMW) glutenin subunits, important determinants of the bread-making quality of wheat flour, have been transformed into wheat, demonstrating that genetic engineering can improve the functional properties of wheat (Blechl and Anderson, 1996; Barro *et al.*, 1997).

Until recently, it has been generally assumed that increases in yield potential in plants are controlled by a large number of genes, each with small additive effects. However, the work of recent years shows that there may also be a few "master genes" that affect the interaction, either directly or indirectly, of several physiological processes that influence yield (Borlaug, 1997). It now appears that the dwarfing genes, *Rht1* and *Rht2* , used to develop the high-yielding Mexican wheats that launched the Green Revolution, also acted as "master genes". At the same time they reduced plant height and improved resistance to lodging, they also increased tillering and the number of fertile florets and the number of grains per spike (Borlaug, 1997). These wheats are short because they respond

atypically to the plant growth hormone gibberellin (Peng JR, *et al.*, 1999). Biotechnology may be a new window through which to search for new "master genes" for high yield potential by eliminating the confounding effects of other genes (Borlaug, 1997).

Commercially, crop biotechnology has made huge impact on global agriculture. In 2003, seven million farmers in 18 countries had grown biotech crops (CropBiotech Net 2004). Biotech crops acreage in 2004 had reached over 180 million acres, including over 100 million acres of the Roundup Ready Soy bean, 40 million acres of biotech corn (Bt and Roundup Ready), 25 million acres of biotech cotton (Bt and Roundup Ready) and 15 million acres of Oil Seed Rape (Roundup Ready) (CropBiotech Net 2004). In the largest biotech crop growth country, US, application of crop biotechnology has already brought immense benefits to farmers, environment and society in general. Growth of the four biotech crops (mentioned above) in US has meant a yearly 46 million pounds less application of pesticides and herbicides, 4 billion pounds of food, feed and fibre production and over $1.5 billion of economic returns (Gianessi *et al* 2002). Undoubtedly, biotech crops is playing an increasingly important role in addressing the world's food security issues, particularly in the developing countries.

1.3 Plant transformation

Many problems of basic and applied aspects in plant biology can be approached by the exploitation of transformation techniques. In general, there are two approaches for plant transformation: *Agrobacterium*-mediated gene transfer and direct gene delivery. The *Agrobacterium*-mediated transformation system is a natural DNA delivery system, which has been very successful for introducing foreign DNA into dicotyledonous plants. However this system has not worked well for most monocotyledonous species. Therefore direct gene delivery systems have been developed. In fact all of the world's most important cereal crops including maize, rice, barley and wheat were first transformed in

this way. This situation has changed in recent years after many years of determined efforts in many laboratories world-wide, and the successful use of *Agrobacterium* have been reported in rice, maize, wheat and barley (see the references below).

1.3.1 *Agrobacterium*-mediated gene transfer

Agrobacterium tumefaciens is a soil bacterium that contains a tumour-inducing plasmid (pTi). Ti plasmids are large, circular double-stranded DNA molecules of about 200 kb. They exist in *Agrobacterium* cells as independently replicating genetic units. *A. tumefaciens* can infect plant cells, causing a crown gall or tumour at or close to the infection site. The tumorous transformation of plants by *Agrobacterium* results from the stimulation of plant cell division by gene products encoded by a segment of DNA (T-DNA) transferred from the bacterium to the plant (reviewed by Zupan and Zambryski, 1995; Tinland, 1996; Zupan *et al.*, 2000). The T-DNA genes of *Agrobacterium tumefaciens* encode enzymes for auxin and cytokinin biosynthesis, and they are responsible for the tumour phenotype (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; Buchmann *et al.*, 1985; Inze *et al.*, 1984; Schroder *et al.*, 1984; Thomashow *et al.* 1984). The T-DNA is delimited by 25-bp imperfect repeats (known as the right and left borders, RB and LB, respectively) that flank the T-DNA (Wang *et al.*, 1984; Sheng and Citovsky, 1996; Tinland, 1996). Any DNA between these borders will be transferred to a plant cell. **Fig 1-1** shows a schematic diagram of the T-DNA transfer from *Agrobacterium* to the plant genome (figure adopted from Veluthambi *et al.*, 2003).

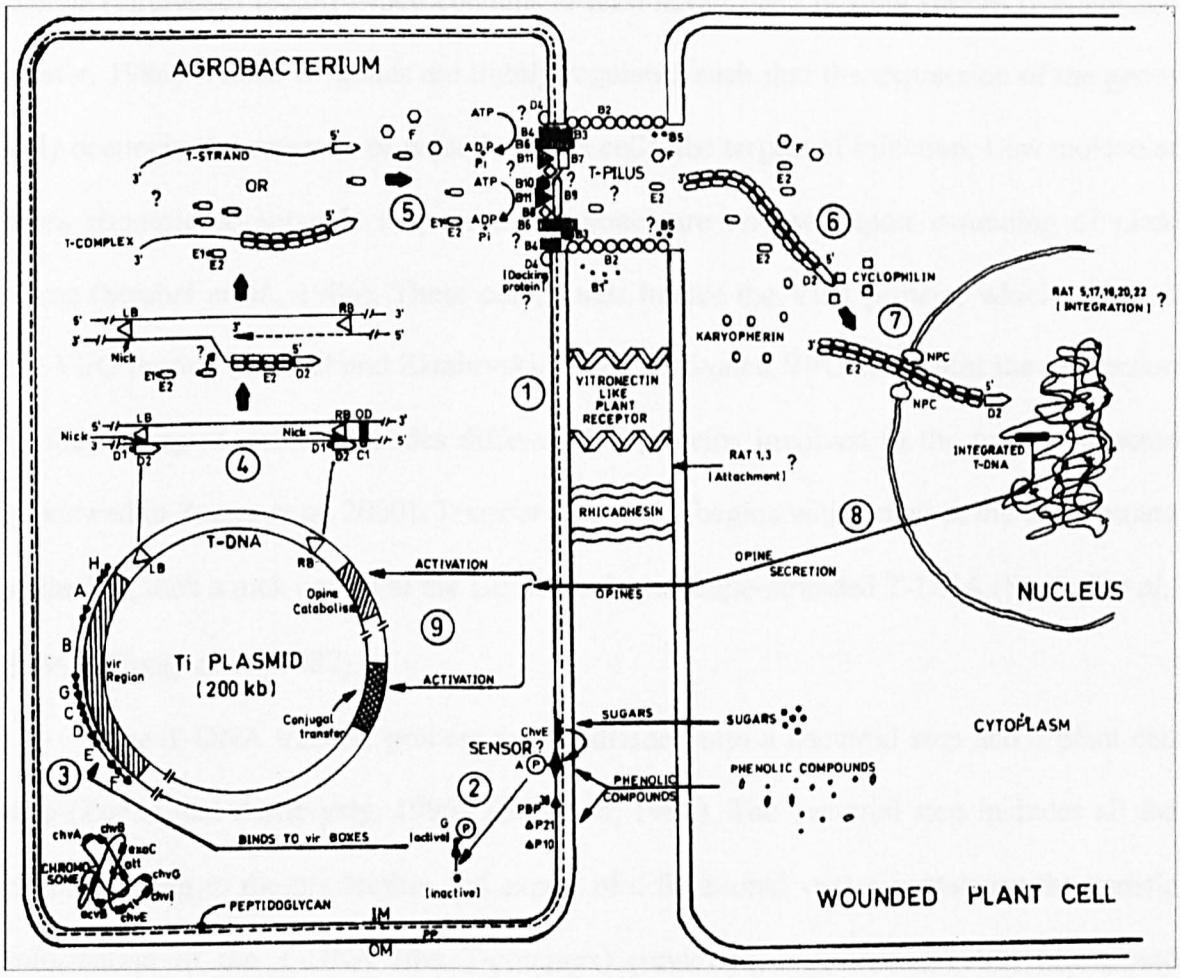


Fig 1-1 A schematic diagram depicting the cascade of events leading to T-DNA transfer from *Agrobacterium* to the plant genome. Steps 1 through 9 indicate sequential processes that occur during infection of a plant cell with *Agrobacterium*. Step 1, binding of *Agrobacterium* to the host cell surface receptors; step 2, direct or indirect mode of recognition of plant signal molecules by the bacterial Vir A/VirG two-component sensortransducer system; step 3, induction of bacterial *vir* genes situated on the Ti plasmid; step 4, generation of the T-strand; step 5, formation of the Tcomplex and its transport into the host plant cell through the T-pilus; step 6, recognition and interaction of T-complex with host cell cytoplasmic proteins; step 7, nuclear transport of T-complex; step 8, T-DNA integration and opine secretion; and step 9, activation of bacterial opine catabolism genes and Ti plasmid conjugal transfer genes. *acvB*, *att*, *chvA*, *chvB*, *chvE*, *chvG*, *chvI* and *exoC* are the chromosome-encoded virulence genes. '?' Indicates that precise steps are yet to be established. P10, P21 and PBP are chromosome-encoded putative phenol-binding proteins. OM, outer membrane; PP, periplasm; IM, inner membrane. Broken lines denote peptidoglycan cell wall. Figure and legend adopted from Veluthambi *et al.* (2003)

The processing and transferring of the T-DNA is mediated by products encoded by the *vir* (virulence) region which contains at least seven open reading frames (Stachel and Nester, 1986). Those *vir* genes are tightly regulated such that the expression of the genes only occurs in the presence of wounded plant cells, the targets of infection. Low molecular mass phenolic compounds (e.g. acetosyringone) are released upon wounding of plant tissue (Stachel *et al.*, 1986). These compounds induce the VirA protein, which activates the VirG protein (Stachel and Zambryski, 1986). Activated VirG stimulates the expression of the *vir* region, which encodes different Vir proteins involved in the transfer process (reviewed in Zupan *et al.* 2000). Transfer of T-DNA begins with a nick in the DNA strand in the RB, then a nick occurs at the LB producing a single-stranded T-DNA (Stachel *et al.*, 1986b; Wang *et al.*, 1987).

The T-DNA transfer process can be divided into a bacterial step and a plant cell step (Zupan and Zambrysly, 1995; Tinland B, 1996). The bacterial step includes all the events leading to the production and export of a functional vector containing the genetic information of the T-DNA (the T-complex) (reviewed in Christie, 1997; Sheng and Citovsky, 1996). The plant cell step includes those events occurring within the plant cell up to the point of the integration of bacterial DNA into the nuclear genome (reviewed in Tinland, 1996; Zupan *et al.* 2000). The T-complex is composed of nucleoprotein (Howard and Citovsky, 1990). It is basically made up of a single-stranded T-DNA molecule (Tinland *et al.*, 1994; Yusibov *et al.*, 1994) associated with at least two types of virulence protein – VirD2 and VirE2. The VirD2 and VirD1 proteins excise the single-stranded T-DNA from the Ti plasmid. The VirD2 protein is a site-specific endonuclease, which recognises and cuts the T-DNA borders (Filichkin and Gelvin, 1993). Upon cutting, VirD2 attaches itself covalently by a phosphotyrosine bound to the 5'-end of the single-stranded T-DNA transferred to the plant cell. The VirE2 protein binds to single—stranded DNA,

and thus it coats the single-stranded T-DNA, protecting it against nucleases in the plant cell (Rossi *et al.*, 1996). Both the VirD2 and VirE2 proteins are believed to target the T-DNA to the plant cell nucleus (Citovsky and Zambryski, 1993), where it is integrated into the plant genome. A *vir*–specific apparatus, the T-complex transporter (T-transporter), mediates transfer of the T-complex from the bacterium to the plant cell. It is assembled from 11 proteins encoded by the *vir*B operon, and VirD4 (reviewed by Zupan *et al.*, 1998; Zupan *et al.*, 2000). The T-transporter facilitates transfer of the T-complex to the plant. It can also transfer proteins, such as VirE2 and VirF, to plant cells (reviewed in Christie, 1997; Zupan *et al.*, 1998). Inside the plant cell, the T-complex is imported into the nucleus where the T-strand becomes stably integrated into a plant chromosome (reviewed in Sheng and Citovsky, 1996; Zupan *et al.*, 2000). Genetically, VirD2 is required for nuclear targeting and faithful integration of the 5' end and VirE2 for the 3' end of the T-strand (Zupan and Zambryski, 1997).

The *Agrobacterium*'s mechanism for T-DNA delivery into plant cells is reasonably well understood. However, not much is known about the role of plant factors in T-DNA transfer and integration within the plant cell. There are several steps in which plant genes are likely to be involved in the *Agrobacterium*-mediated transformation process (Mysore *et al.*, 2000). First, plant-encoded factors could be involved in the initial step of bacterial attachment to the plant cell surface. The next step is the transfer of a T-strand from the bacterium to the plant cells across the plant cell wall and membrane. Once the T-DNA /T-complex enters the cytoplasm of the plant cell, plant factors are required to transport the T-complex to the nucleus. Ballas and Citovsky (1997) showed that a plant karyopherin α (AtKAP α) can interact with VirD2 nuclear localization sequences (NLS) in a yeast two-hybrid interaction system and presumably is involved in nuclear translocation of the T-complex. Other evidence for the involvement of plant factors in T-DNA transfer and

integration comes form the identification of several ecotypes and mutants of *Arabidopsis* that are resistant to *Agrobacterium* transformation (Mysore *et al.*, 2000). They reported the characterization of a T-DNA tagged *Arabidopsis* mutant, *rat5* (resistant to *Agrobacterium* transformation), that is recalcitrant to *Agrobacterium* root transformation. They showed that in *rat5* a histone H2A gene is disrupted, and the T-DNA integration step of transformation is blocked in the *rat5* mutant. Overexpression of *RAT5* in wild-type plants increased *Agrobacterium* transformation efficiency. They hypothesize that histone H2A (*RAT5*) plays an important role in the integration of T-DNA into the plant genome.

Scientists "disarm" the Ti plasmid by eliminating the crown gall disease-causing genes from the T-DNA while leaving the DNA transfer mechanism intact. The two main components for successful *Agrobacterium*-mediated gene transfer, the T-DNA and the *vir* region, can reside on separated plasmids. These form the basis of modern Ti plasmid vectors, termed binary Ti vectors (Hoekema *et al.*, 1983; Bevan 1984). The *vir* gene functions are provided by the disarmed Ti plasmids resident in the *Agrobacterium* strain. The T-DNA, within which are the genes to be transferred, is provided in the vector. The genes of interest, together with a selectable marker, are inserted in the binary vector T-DNA region so that these foreign genes can be transferred into the plant genome (Stachel *et al.*, 1985). The development of the leaf disk technique (Horsch *et al.*, 1985) provided a simple but very efficient method for *Agrobacterium*-mediated gene transfer.

1.3.2 Direct gene transfer

All of the world's most important cereal crops: maize, rice, barley and wheat, were first transformed by direct gene transfer – the exploitation of a physical means of DNA delivery into plant cells rather than a biological approach.

Direct gene transfer systems such as electroporation (Fromm *et al.*, 1986), direct DNA uptake (Lorz *et al.*, 1985), liposome fusion (Fraley *et al.*, 1982), and microinjection

(Crossway *et al.*, 1985) generally require the enzymatic removal of cell walls. Rice was the first major cereal crop to be transformed after direct gene transfer into regenerable protoplasts (Toriyama *et al.*, 1988; Zhang *et al.*, 1988; Zhang and Wu, 1988; Shimamoto *et al.*, 1989; Datta *et al.*, 1990), and maize followed (Rhodes *et al.*, 1988). However, this approach relies heavily upon the establishment of regenerable cell suspension cultures, which is highly empirical, genotype-dependent and time-consuming (Vasil *et al.*, 1990). In contrast, intact plant cells in suitable tissue/organ types can regenerate into whole plants more readily in cereals and grasses (Vasil *et al* 1982). Therefore, a simpler tissue culture system using intact immature embryos or young inflorescences as the target tissue was used for successful transformation. Transgenic plants have been produced by using immature embryos as target tissues and electroporation for DNA delivery (D'Halluin *et al.*, 1992; Ke *et al.*, 1997; He *et al.*, 1998; Barro *et al.*, 1999; Sorokin *et al.*, 2000).

The invention of the "particle gun" has provided another powerful tool for cereal transformation (Klein *et al.*, 1987; Klein *et al.*, 1988). It can efficiently deliver DNA directly into intact cells and tissues possessing the potential for regeneration into whole plants. DNA is delivered to plant cells in association with microscopic metal particles, called microcarriers. Both tungsten particles and gold particles are used as microcarriers of DNA. Transgenic maize (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990), rice (Christou *et al.*, 1991; Cao *et al.*, 1992), barley (Wan and Lemaux, 1994), and wheat (Vasil *et al.*, 1992; Weeks *et al.*, 1993; Vasil *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994) were produced this way. So far, this method has proven to be the most robust method for transformation of cereals. There are some other methods such as pollen tube, microinjection and viral vector (agroinfection), but they are not reproducible (reviewed in Ingram *et al.*, 2001).

1.3.3 Cereal transformation systems

To date, particle bombardment is still the most successful method for wheat and barley transformation. Target genes and selectable markers/reporters can be put in different plasmid constructs and co-transformed into plants. The frequencies of co-transformation are usually very high.

Agrobacterium-mediated wheat and barley transformation systems have been recently developed as an attractive alternative method for genetic transformation of wheat and barley (Cheng *et al.*, 1997; Tingay *et al.*, 1997; Guo *et al.*, 1998; Wu *et al.*, 1998; Weir *et al.*, 2001). There are also a few reports of wheat transformation using other DNA delivery methods, such as electroporation (Ke *at al.*, 1997; Sorokin *et al.*, 2000) and silicon carbide fibres (Serik *et al.*, 1996).

Agrobacterium-mediated gene transfer has several advantages over direct gene delivery. It can transfer relatively large segments of DNA with little rearrangement, and insert the segments into the recipient genome at a fairly low copy number (Tinland, 1996). Although monocotyledons are not the natural hosts of *Agrobacterium tumefaciens*, extensive efforts have been undertaken to investigate the potential for transformation of monocotyledons, especially cereals, *via A. tumefaciens*. In nature, dicotyledonous plant cells become susceptible to *Agrobacterium* when they are wounded. These wounded plant cells produce abundant signal molecules such as acetosyringone (AS) and hydroxyacetosyringone (OH-AS) which activate T-DNA transfer from *A. tumefaciens* (Stachel *et al.*, 1985). Therefore acetosyringone (AS), or a cell suspension culture from plants susceptible to *Agrobacterium*, is usually added as a supplement to the inoculation and co-cultivation media in *Agrobacterium*-mediated cereal transformation experiments to increase the chance of success (Chan *et al.*, 1993; Hiei *et al.*, 1994).

In recent years, significant progress has been made in *Agrobacterium*-mediated

cereal transformation. Chan *et al.* (1993) first reported the production of transgenic rice plants by inoculating immature embryos with an *A. tumefaciens* strain, and they found that co-incubation of a potato suspension culture (PSC) with the *Agrobacterium* inoculum significantly improved the transformation efficiency of rice. Hiei *et al.* (1994) obtained a large number of transgenic rice plants by *Agrobacterium*-mediated transformation. The results from Hiei *et al.* (1994) show that the addition of acetosyringone to the co-cultivation medium is essential. The use of 'super-virulent' *Agrobacterium* strains, the development of 'super-binary' vectors also facilitate the process (Hiei *et al.*, 1994). A281 is a so-called 'super-virulent' strain of *A. tumefaciens,* because it carries a "super-virulent" Ti plasmid, pTiBo542, and its host range is wider and transformation efficiency is higher than those of other strains (Jin *et al.*, 1987; Komari, 1989). Since strains that carried pTiBo542 operate very efficiently in transformation, two new types of system based on pTiBo542 have been developed (Hiei *et al.*, 1994). The first involves strain EHA101 (Hood *et al.*, 1986), which carries a 'dis-armed' version of pTiBo542, and this strain has been popular in trials aimed at transformation of monocotyledons. The second involves what is known as a 'super-binary' vector in which a DNA fragment from the virulence region of pTiBo542 is introduced into a small, T-DNA-carrying plasmid (Komari, 1990) used in a binary vector system.

To date, successful *Agrobacterium*-mediated gene transformation has been reported in major cereal crops including rice (Chan *et al.*, 1993; Hiei *et al.*, 1994; Dong *et al.*, 1996; Zhang *et al.*, 1997), maize (Ishida *et al.*, 1996), barley (Tingay *et al.*, 1997; Wu *et al.*, 1998) and wheat (Cheng *et al.*, 1997; Pukhalskii *et al.*, 1996; Weir *et al.*, 2001). However, only rice transformation by this approach has become routine, allowing application in both gene testing and crop improvement. In wheat and barley, confirmation of the initial successes is still awaited.

1.4 Current status of wheat and barley transformation

Although wheat and barley were the last of the economically important cereals to be transformed (Vasil *et al.*, 1992; Wan and Lemaux, 1994), the subsequent progress of wheat and barley transformation has been remarkable. Transgenic wheat and barley plants have been produced in a number of laboratories (Weeks *et al.*, 1993; Vasil *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994; Wan and Lemaux, 1994; Blechl and Anderson, 1996; Barro *et al.*, 1997; Tingay *et al.*, 1997; Cheng *et al.*, 1997; Stoger *et al.*, 1998; Chen *et al.*, 1998; Bliffeld *et al.*, 1999). The research emphasis has been shifted from transformation methodology towards its application. Now it is possible to transfer economically important traits into commercial varieties.

1.4.1 Donor plants and tissue cultures

Genotypes

In most experiments the cultivars used for transformation were limited to "model" plants, which respond well to tissue culture *in vitro*, rather than varieties of agronomic importance. Cereal tissue culture response is highly genotype dependent. Takumi and Shimada (1997) examined the culture response of immature embryos *in vitro* and production of transgenic plants of six common wheat cultivars. They found that the frequency of stable transformation varied with the genotype; this was due to the difference in the culture response of the genotype *in vitro*, rather than the efficiency of the introduction of the transgene into wheat cells by particle bombardment. Therefore, potential commercial cultivars for transformation can be identified by screening genotypes with a high regeneration capability (Machii *et al.*, 1998; Iser *et al.*, 1999).

Explants

Among the successful transformation protocols, the target tissues are usually fresh

immature zygotic embryos, precultured immature embryos, immature embryo-derived embryogenic calli, or microspores. Isolated scutella from immature embryos of wheat and barley are commonly used as targets for genetic transformation. Barro *et al.* (1999) compared the frequencies of regeneration from immature inflorescences and immature scutella of elite cultivars of wheat, barley and Tritordeum. They found, in wheat, that shoot regeneration from scutella was markedly higher than from inflorescences.

Recently, Pasternak *et al.* (1999) reported a new approach to obtaining embryogenic callus and regenerated plants. They established a reproducible culture system for embryogenic callus formation and plant regeneration from leaf base segments of barley. They found that a high zinc level in the medium used for germination of the donor seed resulted in an extension of the basal leaf sector from which callus was formed. Since multiple plants can be regenerated by culture of leaf segments derived from seedlings grown *in vitro*, there is no need to produce donor plants in the greenhouse.

Another potential target for cereal transformation is the flower pistil. Pukhalskii *et al.* (1996) produced transgenic wheat plants by applying *Agrobacterium* cells to flower pistils after artificial pollination and the transformation frequency was at least 2.7%. Ye *et al.* (1999) demonstrated that when using *Agrobacterium* to transform *Arabidopsis* floral organs (*in planta* transformation), ovules were targets for receiving T-DNA..

Tissue cultures

In most protocols, callus induction media are similar, especially with regard to the plant growth regulators. In wheat, for example, 2,4-dichlorophenoxyacetic acid (2,4-D) is commonly used for callus induction. Barro *et al.* (1998) compared the effects of different auxin regimes, picloram and 2,4-D, in a population of transgenic wheat and tritordeum lines. They found that the presence of picloram results in higher transformation efficiencies than the presence of 2,4-D.

It has been reported that the immature embryos from plants that had been regenerated *via* tissue culture procedures, respond well to procedures for callus formation and plant regeneration (Harvey *et al.*, 1999). There is a lack of detailed understanding of the regeneration pathway in cereals, particularly how and where the somatic embryos are formed. A better understanding of this part of the method will enhance further the efficiency of the transformation procedures.

1.4.2 Genes involved in wheat and barley transformation

Reporter genes

Plasmid constructs for wheat and barley transformation usually contain the reporter β-glucuronidase gene (*gusA*) as a reporter. The expression of the *gusA* gene can be analyzed histochemically using 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) (Jefferson *et al.*, 1987). Gene delivery systems can be assessed and optimised by analyzing GUS activity. Other reporter genes such as chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase II (NPTII) have also been used for gene expression (Topfer *et al.*, 1988). However the assays of enzymatic activities of CAT, NPTII and GUS are destructive, therefore they cannot be used to monitor gene expression *in vivo*.

Recently, several visual reporter genes have been tested and applied in plant transformation. These visual reporters include anthocyanin regulatory genes (Ludwig *et al.*, 1989, in maize; Goff *et al.*, 1990, in maize; Bower *et al.*, 1996, in sugarcane; McCormac *et al.*, 1998a, in wheat and barley; Wu *et al.*, 1998, in barley; Chawla *et al.*, 1999, in wheat); the firefly luciferase gene (Ow *et al.*, 1986; Lonsdale *et al.*, 1998a, in wheat and maize, Lonsdale *et al.*, 1998b, in wheat; Mudge and Birch, 1998, in tobacco; Baruah Wolff *et al.*, 1999, in rice); and the jellyfish green fluorescent protein gene (Chiu *et al.*, 1996; Reichel *et al.*, 1996; Pang *et al.*, 1996; Jordon, 2000; Weir *et al.*, 2001). The green fluorescent protein (GFP) gene can be used as a non-destructive reporter and is

suitable for monitoring gene expression *in vivo*. There are several versions of *gfp* with improved expression. The *gfp* gene is now widely used in plant transformation as a vital screenable and selectable marker, and it has been transformed into cells/plants of all major cereal crops including maize (Chiu *et al.*, 1996; Pang *et al.*, 1996; van der Geest and Petolino, 1998; Elliott *et al.*, 1999); rice (Nagatani *et al.*, 1997; Vain *et al.*, 1998; Xu *et al.*, 1998; Jang *et al.*, 1999); oat (Kaeppler *et al.*, 2000); barley (McCormac *et al.*, 1998a; Ahlandsberg *et al.*, 1999) and wheat (Pang *et al.*, 1996; McCormac *et al.*, 1998a; Jordan, 2000; Weir *et al.*, 2001). The non-destructive exploitation of visual markers, such as *gfp* and *luc*, forms part of a strategy for using them to study the regeneration pathway to production of transgenic plants without using the conventional selectable markers.

Selectable markers

Primary screens for transformed plants have almost exclusively relied upon antibiotic/herbicide-based selection. Commonly used selectable markers include the phosphinothricin acetyltransferase gene (*bar*), the neomycin phosphotransferase II gene (*nptII*) and the hygromycin phosphotransferase gene (*hpt*), which allow the transformants to survive under herbicide or antibiotic selection. The most widely used selectable marker is the *bar* gene which facilitates selection with bialaphos or phosphinothricin (PPT). PPT, the active component of the herbicides Basta and Challenge, is a strong inhibitor of glutamine synthetase (Thompson *et al.*, 1987). PPT is commonly used in wheat transformation, and bialaphos is used to select transgenic barley plants. Altpeter *et al.* (1996b) found that bialaphos is more efficient than PPT in wheat transformation.

Witrzens *et al.* (1998) compared three selectable markers (*bar*, *aphA* and *hpt*) in wheat. Transformed plants were recovered using the *bar* gene in combination with bialaphos, and the *aphA* (neomycin phosphotransferase) gene in combination with geneticin or paromomycin; but no transgenic material was obtained with the *hpt*

(hygromycin phosphotransferase) gene and hygromycin B. On the contrary, Ortiz *et al.* (1996) claimed the hygromycin resistance gene was an efficient selectable marker for wheat transformation, and they concluded that the *hpt* gene was as good as or a better than the *bar* gene.

The use of antibiotic resistance genes as markers for the selection of transgenic crops has resulted in the particular concern that these genes may be transferred into pathogenic bacteria. While these concerns are unlikely to be justified, it's necessary to develop more benign markers for transformation. Recently, a new selectable marker system, the MAT (Multi-Auto-Transformation) vector system, for plant transformation has been developed (Ebinuma *et al.*, 1997; Ebinuma *et al.*, 2001). The system is distinctive in its combination of the *ipt* gene and removable elements which remove the introduced *ipt* gene from plant cells. Two kinds of MAT vectors have been reported: (1) the *ipt*-type MAT vector, in which the isopentenyl transferase (*ipt*) gene of *A. tumefaciens* PO22 is used as a selectable marker for regenerating transgenic cells and selecting marker-free transgenic plants; (2) the *rol*-type MAT vector, in which the 7.6-kb *Eco*RI fragment containing the *rol* A, B. C genes of *A. rhizogenes* NIAES1724 is used (reviewed in Ebinuma *et al.*, 1997; Ebinuma *et al.*, 2001). The *ipt* gene codes for the isopentenyl transferase that catalyses cytokinin synthesis and causes the proliferation of transgenic cells and the differentiation of adventitious shoots. The *rol* genes are responsible for the proliferation of 'hairy roots' by increasing auxin sensitivity. In the MAT vectors, the chimeric *ipt* gene or the *rol* genes are combined with the maize transposable *Ac* element or the site-specific recombination *R*/RS system to remove them from the transgenic cells after transformation (Ebinuma *et al.*, 2001).

Wright *et al.* (2001) reported the use of the phosphomannose isomerase gene, *pmi*, as a selectable marker for the transformation of maize and wheat. The selectable marker

consists of the *manA* gene from *Escherichia coli* that encodes phosphomannose isomerase, *pmi*, under the control of a plant promoter. Only the transgenic plants were able to metabolise the selection agent, mannose, into a usable source of carbon, fructose. Wright *et al.* (2001) found that using the selectable marker *pmi* with mannose-containing selection media gave high transformation frequencies and few escapes. They also found that *pmi* performed better than *bar* under the conditions they tested.

Target genes

It is now possible to transfer economically important traits into cereals. These important traits include grain quality and resistance to diseases and insects.

In wheat, high-molecular-weight glutenin subunits (HMW-GS) are important determinants of the bread-making quality of wheat flour. HMW-GS genes have been transformed into wheat by several groups to obtain transgenic wheat plants that express a novel high-molecular-weight glutenin subunit (Shewry *et al.*, 1995; Blechl and Anderson, 1996; Altpeter *et al.*, 1996a; Barro *et al.*, 1997; Shimoni *et al.*, 1997; Blechl *et al.*, 1998; Rooke *et al.*, 1999). Another example of changing grain quality is starch modification. Baga *et al.* (1999) developed various monocotyledon vectors that drive expression of wheat starch branching enzyme I (SBEI) cDNA sequences in the anti-sense orientation. One transgenic wheat plant expressing the anti-sense SBEI RNA produces a ten-fold lower level of branching enzyme (BE) activity in kernels than wild type wheat. Analysis of starch produced from the transgenic plant shows that starch structure and properties have been altered.

Several disease resistance genes have been transformed into wheat and barley (Bushnell *et al.*, 1998). These genes include the gene encoding the barley yellow mosaic virus coat protein (Karunaratne *et al.*, 1996, in wheat); stilbene synthase genes (for increased fungal resistance) (Brauer *et al.*, 1997, in wheat and barley; Leckband and Loze,

1998, in wheat and barley; Fittig and Hess, 1999, in wheat); the chitinase gene (for increased resistance to powdery mildew disease) (Chen *et al.*, 1998, in wheat; Bliffeld *et al.*, 1999, in wheat); and the rice thaumatin-like protein (TLP) gene (*tlp*) (for controlling wheat scab symptoms) (Chen *et al.*, 1999, in wheat).

Genes for increased insect resistance have also been transformed into wheat. Altpeter *et al.* (1999) introduced the barley trypsin inhibitor Cme (BTI-CMe) into wheat. Stoger *et al.* (1999) produced transgenic wheat plants containing the gene encoding snowdrop lectin for enhanced resistance to the grain aphid.

1.5 Objectives of this study

Our lack of understanding of the cellular basis of wheat somatic embryogenesis requires the investigation of the plant regeneration pathway, which is described in Chapter Three. Wheat transformation is described in Chapter Four, while barley transformation is in Chapter Five.

The lengthy selection period required when using conventional markers suggests that new visual marker genes should be exploited in wheat and barley transformation. This investigation is presented in Chapters Four (*sgfp* and *C1/Lc*) and Chapter Five (*luc*).

Agrobacterium-mediated gene transfer has several advantages in comparison with the direct gene delivery (see above section 1.3.3). However, at the time this project was conducted, the only successful gene delivery method for wheat and barley was microprojectile bombardment. Therefore *Agrobacterium*-mediated transformation as an alternative method for DNA delivery was tested in wheat, and this research is described in Chapter Four (section 4.5).

Chapter Two

METHODOLOGY

2.1 Plant materials and growth conditions

2.1.1 Wheat

Commercial winter and spring wheat varieties (*Triticum aestivum L.*) were used in this study. The winter wheats include Buster, Dynamo, Kedong 58, Flame, Spark, Wasp and Zodiac, and spring wheats include Rascal, Scamp and Tonic. All seeds were bought from Nickerson Seed Company except Kedong 58 (a Chinese variety, which was obtained from Professor H. Hu). Winter varieties were grown in the field in late autumn and the immature embryos were collected and cultured the next summer. These materials did not respond well in tissue culture. Therefore their results were not included in the thesis. The poor response of these winter wheats might be due to the hot weather when the materials were available, as we found later that the growth conditions of the donor plants are critical: material harvested in early spring in the greenhouse responded better than that harvested in the summer.

The seeds of spring wheat Rascal, Scamp and Tonic were sown in batches with an interval of three weeks. The plants were grown in a glasshouse maintained at a minimum of 12 °C and with natural daylight supplemented by high-pressure mercury vapour discharge lamps (400W) to provide a 16h day-length. Immature seeds were harvested 12 to 18 days after anthesis (when the immature embryos was about 1.0-1.5 mm in diameter), for tissue culture and subsequent transformation. Transgenic plants were grown under identical conditions in a contained glasshouse.

Kedong 58 is a weak-winter wheat which requires slight vernalization. To grow the winter wheat Kedong 58, seeds were soaked in water in Petri dishes with filter paper, and kept in 4 °C cold room for 3-4 weeks before transfer into soil in a glasshouse. Regenerated plantlets from K58 were vernalised by keeping in a 4 °C cold room with fluorescent light for 3 to 4 weeks before transfer into soil in a glasshouse. They were fully fertile.

2.1.2 Barley

Barley plants (Golden Promise) were grown in controlled environment rooms in the John Innes Centre. The growth conditions have been described by Harwood *et al.* (2000). The temperature in the controlled environment rooms was 15 °C during the day and 12 °C at night, with 80% relative humidity and light levels of 500 $\mu mol.m^{-2}.s^{-1}$. Transgenic barley plants were grown under identical conditions in a contained glasshouse.

2.2 Tissue culture

2.2.1 Media for tissue culture

Media for wheat tissue culture

The following media were used in wheat tissue culture and selection media:

MS-b	Callus induction medium
MS-h	MS-b containing a higher concentration of sucrose (5% to 20%)
MSb-PPT(1)	MS-b supplemented with (1 mg/l) PPT
MS-AS	MS-b supplemented with 100μM acetosyringone (AS)
MS-I	MS-AS supplemented with 500mg/l cefotaxime
MS-r	Plant regeneration medium
PPT(5)	MS-r supplemented with (5mg/l) PPT

The callus induction medium MS-b consisted of MS basic medium (Murashige and Skoog, 1962), supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l d-biotin, 100 mg/l casein hydrolysate, 3% sucrose and solidified with 0.3% gelrite. MS-h medium was the same as MS-b except that it contained a higher concentration of sucrose (5% to 20%), which was used to cause osmotic changes before particle bombardment (Vain *et al.*, 1993). Medium MS-AS was MS-b medium supplemented with 100μM acetosyringone (AS) for co-cultivation of wheat tissues and bacteria, and medium MS-I

was supplemented with 500mg/l cefotaxime in order to inhibit bacterial growth after co-cultivation.

The regeneration medium MS-r consisted of MS basic medium supplemented with 0.5 mg/l NAA, 0.5 mg/l Kinetin, 3% sucrose and solidified with 0.3% gelrite. Phosphinothricin (PPT) was used for *bar* gene selection. Media PPT1, PPT3 and PPT5 are MS-r medium containing 1 mg/l, 3 mg/l and 5 mg/l PPT respectively. 1mg, 3mg or 5mg of PPT was added to 1 litre of MS-b medium, forming MSb-PPT medium for early herbicide selection.

Media for barley tissue culture

The following media were used for barley tissue culture and selection:

BCI	Callus induction medium
BCI5B	BCI medium supplemented with 5 mg/l bialaphos
BCI+0.4M	BCI medium supplemented with 0.4M mannitol
FWG	Plant regeneration medium
DBC3B	FWG medium with high $CuSO_4$ (5μM), 1 mg/l 2,4-D and 3 mg/l bialaphos
FWG1B	FWG medium supplemented with 1 mg/l bialaphos
BCI1B	BCI medium without dicamba but supplemented with 1 mg/l bialaphos

Callus induction medium (named BCI medium) contains the basal salts of MS medium (Murashige and Skoog 1962), supplemented with 350 mg/l myo-inositol, 69 mg/l proline, 1 mg/l thiamine-HCl, 1 g/l casein hydrolysate, 2.5 mg/l dicamba, 30 g/l maltose and solidified with 3.5 g/l phytagel. For plant regeneration, medium FWG was used. It contained the basal salts of MS medium, but only one tenth of NH_4NO_3 , and supplemented with 100 mg/l Myo-inositol, 750 mg/l glutamine, 0.4 mg/l thiamine-HCl, 20 g/l maltose and solidified with 3.5 g/l phytagel.

All stock solutions were filter sterilised and kept in the refrigerator or cold room (4 °C). Media without phytagel were first prepared at double strength, then the pH was adjusted to 5.8 (with KOH) and the solution was filter sterilised. Double strength phytagel (3.5g of phytagel was dissolve in 500ml H_2O in a 1000ml bottle) was autoclaved and stored at room temperature. Double strength medium (filter sterile) and double strength phytagel were pre-warmed in a 65°C water bath and mixed in equal volumes. Filter sterilised plant growth regulator or bialaphos solution (if applicable) was added to the medium at this stage and mixed well before pouring the medium into Petri dishes or tubes.

2.2.2 Immature embryo isolation and culture

Wheat immature embryo isolation and tissue culture

Immature wheat seeds were collected 12 to 18 days post-anthesis and surface sterilised in 10-20% (v/v) Domestos (containing 0.5% - 1.0% sodium hypochlorite) for 10-20 minutes, followed by five rinses with sterile distilled water. The immature embryos were isolated and cultured on MS-b medium (25-30 immature embryos per dish) with their scutella sides facing up. The embryos were cultured in the dark (covering the Petri dishes with foil) in a 24°C incubator. Two to four weeks after callus induction, embryogenic calli were transferred to MS-r medium for regenerating shoots and roots, and cultured under light.

For transformation experiments, immature embryos were pre-cultured for 1-7 days before microprojectile bombardment. Then immature embryos or calli were transferred to MS-h medium for osmotic treatment for 2-6 hours before particle bombardment. They remained on this medium for one day before transfer to fresh MS-b medium. The cultures bombarded with plasmid pDB1 or pAHC25 were transferred to selection medium PPT1 or PPT5 after 1-3 weeks, then transferred to fresh PPT5 medium every 2-4 weeks.

Barley immature embryo isolation and culture

About two weeks after anthesis (when the immature embryos were about 1mm in diameter), spikes were collected and the immature seeds were removed. The seeds were put into a jar, washed briefly (about 1 minute) in 70% ethanol and rinsed with sterile distilled water. Then the seeds were soaked in a solution of sodium hypochlorite (6% w/v available chlorine) for 4 minutes followed by three washes with sterile water. The immature embryos were dissected from the immature seeds using a stereo microscope, and the embryonic axes were removed from the embryos using a fine forceps. (Note: a quicker way to remove the embryonic axis involved removal of half of the lemma to expose the embryo, leaving the other half of the lemma in place and holding the embryo (still on the seed); then the tip of a fine forceps (with a curved end) was put into the gap between the axis and the scutellum, and the axis was hooked out in the direction of the coleorhiza). The embryos were incubated on callus induction medium BCI with the scutellum side up, and distributed evenly on the medium (about 100 embryos per 9 cm diameter Petri dish). After covering the dishes with foil, the embryos were cultured in the dark in a 24°C incubator.

2.2.3 Osmotic treatment

Vain *et al.* (1993) reported that osmotic treatment enhanced particle bombardment-mediated transient and stable transformation of maize. They found that cells treated on an osmoticum-containing medium (0.2 M sorbitol and 0.2 M mannitol) 4 h prior to and 16 h after bombardment resulted in a statistically significant 2.7-fold increase in transient beta-glucuronidase expression, and 6.8-fold increase in recovery of stably transformed maize clones. They believe the basis of osmotic enhancement of transient expression and stable transformation resulted from plasmolysis of the cells which may have reduced cell damage by preventing extrusion of the protoplasm from bombarded cells.

Wheat immature embryos or calli were transferred to MS-h medium for osmotic treatment 2-6 hours before particle bombardment. They remained on this medium for one day before transfer to fresh MS-b medium.

Barley immature embryos were transferred to another callus induction medium (BCI+0.4M) containing 0.4M mannitol one day after isolation for osmotic treatment. Twenty to thirty embryos were arranged in the centre of each dish (5 cm diameter) with the scutellum face up. They remained on this osmotic treatment medium for 24 hours. Biolistic bombardment was carried out 4 hours after the embryos were transferred onto the osmotic treatment medium.

2.3 Plasmid DNA

Four plasmid constructs, pDB1, pAHC25, pBECKS.sgfp-S65T and pBECKS.red, were used for wheat transformation, and two plasmid constructs, pDAPH7 and pAL51, were used for barley transformation (**Fig. 2-1 to Fig. 2-3**). Plasmids pDB1 and pAHC25 are dual expression vectors containing the *gusA* reporter gene encoding β-glucuronidase, and the selectable *bar* gene (Becker *et al.*, 1994; Christensen and Quail, 1996). Plasmids pBECKS.sgfp-S65T and pBECKS.red are binary vector constructs containing visual reporter genes: a synthetic green fluorescent protein gene (pBECKS.sgfp-S65T) and *C1/Lc* anthocyanin-biosynthesis regulatory genes (pBECKS.red) (McCormac *et al.*, 1998a). Plasmid pDAPH7 contains *dapA* gene (a lysine biosynthesis regulatory gene), whilst plasmid pAL51 contains the *bar* selectable marker and the luciferase (*luc*) reporter gene (Lonsdale *et al.* 1995).

2.3.1 pDB1

Plasmid pDB1 was kindly provided by Professor H. Lorz of the Institute for General Botany, University of Hamburg, Germany. It contains the *gusA* under the control of the

actin-1 promoter and the *bar* under the control of the CaMV 35S promoter (Becker *et al.* 1994) (**Fig. 2-1**).

2.3.2 pAHC25

Plasmid pAHC25 was kindly provided by Professor P. H. Quail at the Department of Plant Biology, University of California, Berkeley. It contains the *bar* and *gusA* genes, each under the control of the maize ubiquitin *Ubi-1* promoter (Christensen and Quail, 1996) (**Fig. 2-1**).

2.3.3 pBECKS.sgfp-S65T

Plasmid pBECKS.sgfp-S65T is a binary vector constructed by Dr A. C. McCormac at The Norman Borlaug Institute for Plant Science Research, De Montfort University, Leicester. It contains a green fluorescent protein (GFP) gene which has been synthesised for altered codon usage and contains a point mutation (Chiu *et al.*, 1996); this gene (*sgfp (S65T)*) was kindly provided by Jen Sheen (MGH, Boston, MA). The *sgfp (S65T)* gene contains the favored codons of highly expressed human proteins, gives 20-fold higher GFP expression in maize leaf cells than the original jellyfish GFP sequence. When combined with a mutation in the chromophore, the replacement of the serine at position 65 with threonine, the new GFP sequence gives more than 100-fold brighter fluorescent signals upon excitation with 490 nm (blue) light, and swifter chromophore formation (Chiu *et al.*, 1996).

In the binary vector pBECKS.sgfp-S65T, the *sgfp (S65T)* gene is fused to a CaMV 35S promoter and nopaline synthase (*nos*) polyadenylation (PA) signals. This 35S:sgfp(S65T) gene was isolated as a *Hin*dIII/*Eco*RI fragment and inserted into the multiple cloning site of $pBECKS_{400}.Z_{sk}$ (a binary vector which is also used for

Agrobacterium-mediated transformation experiments in our laboratory) (McCormac *et al.*, 1997; McCormac *et al.*, 1998a) (**Fig. 2-2**).

2.3.4 pBECKS.red

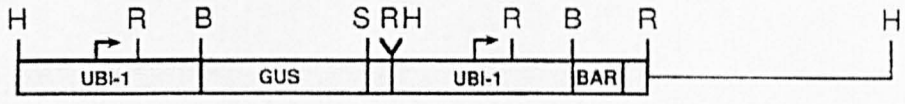
Another binary vector also constructed by Dr A. C. McCormac, plasmid pBECKS.red contains *C1* and *Lc* anthocyanin-biosynthesis regulatory genes. *C1* gene was from plasmid p35S-C1 (Goff *et al.*, 1990) provided by S. A. Goff, and *Lc* gene from pSRL439 (Ludwig *et al.*, 1989) provided by S. R. Wessler. The two genes were inserted into the multiple cloning site of the T-region (McCormac *et al.*, 1998a) (**Fig. 2-2**).

2.3.5 pDAPH7

Plasmid pDAPH7 was prepared in Udine, Italy (an INCO - COPERNICUS programme funded by the EU). It contains the *dapA* gene (lysine gene) under the control of a 35S promoter (**Fig. 2-3**).

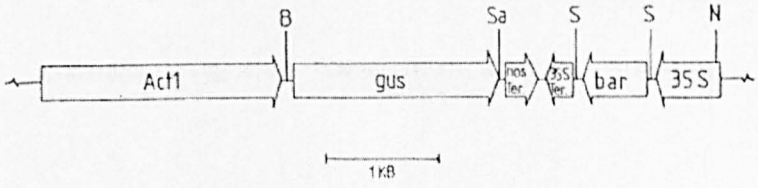
2.3.6 pAL51

Plasmid pAL51 was kindly provided by Dr D. M. Lonsdale of The Department of Molecular Genetics, John Innes Centre. It contains the *bar* selectable marker and the luciferase (*luc*) reporter gene, both under the control of maize ubiquitin (*ubi-1*) promoters (Lonsdale *et al.* 1995) (**Fig. 2-3**).



B=*Bam*HI; H=*Hind*III; R=*Eco*RI; S=*Sac*I

pAHC25



B=*Bam*HI; N=*Nco*I; S=*Sal*I; Sa=*Sac*I

pDB1

Fig. 2-1 Plasmid constructs used in wheat transformation.

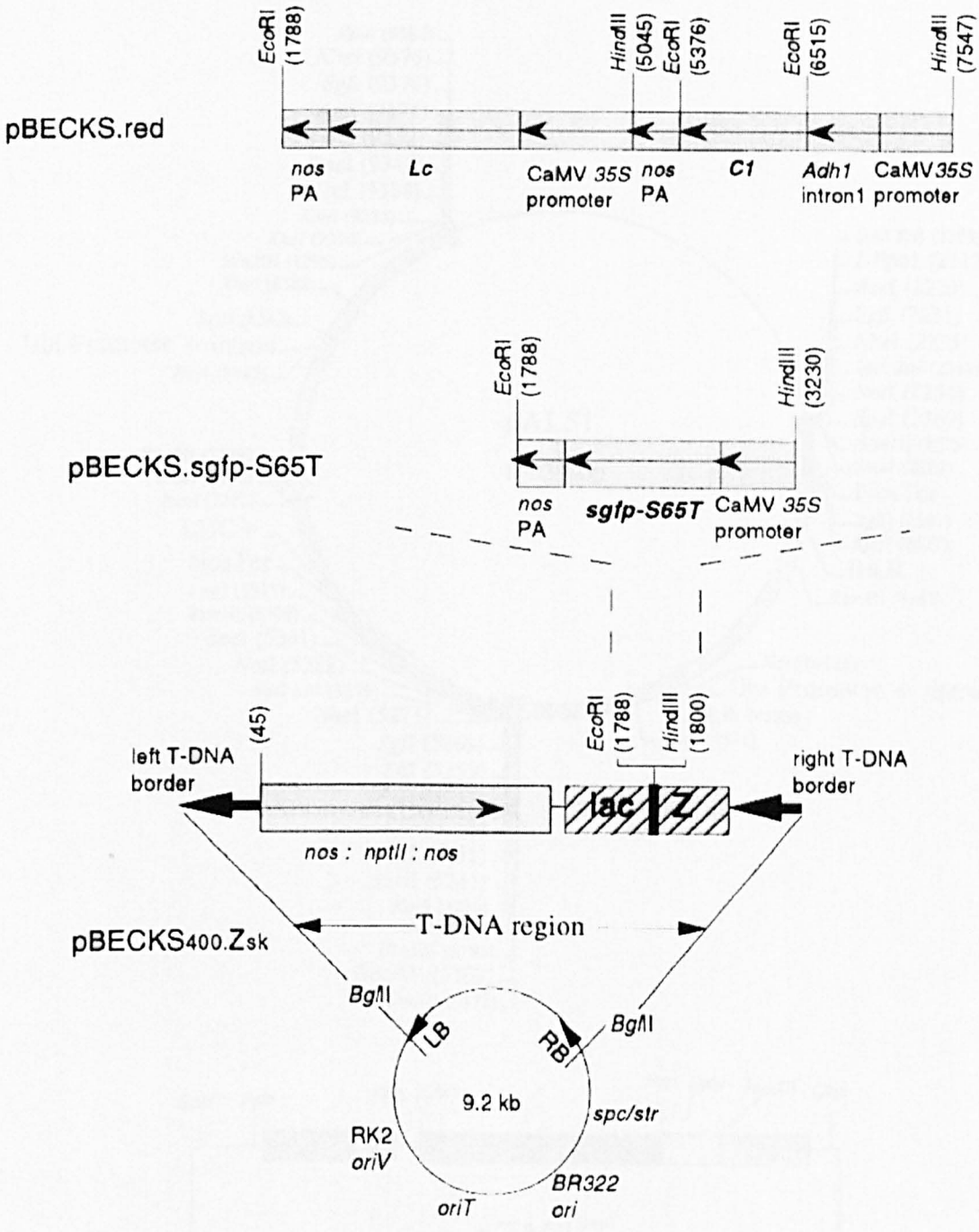


Fig. 2-2 Plasmid constructs used in wheat transformation. Binary T-DNA vectors containing reporter genes for anthocyanin biosynthesis (pBECKS.red) and green fluorescent protein (pBECKS.sgfp-S65T). All constructs were based on an original binary plasmid, $pBECKS_{400}Z_{sk}$ and also contain the *npt*II gene which confers plant resistance to kanamycin or G418 (from McCormac *et al.*, 1998a).

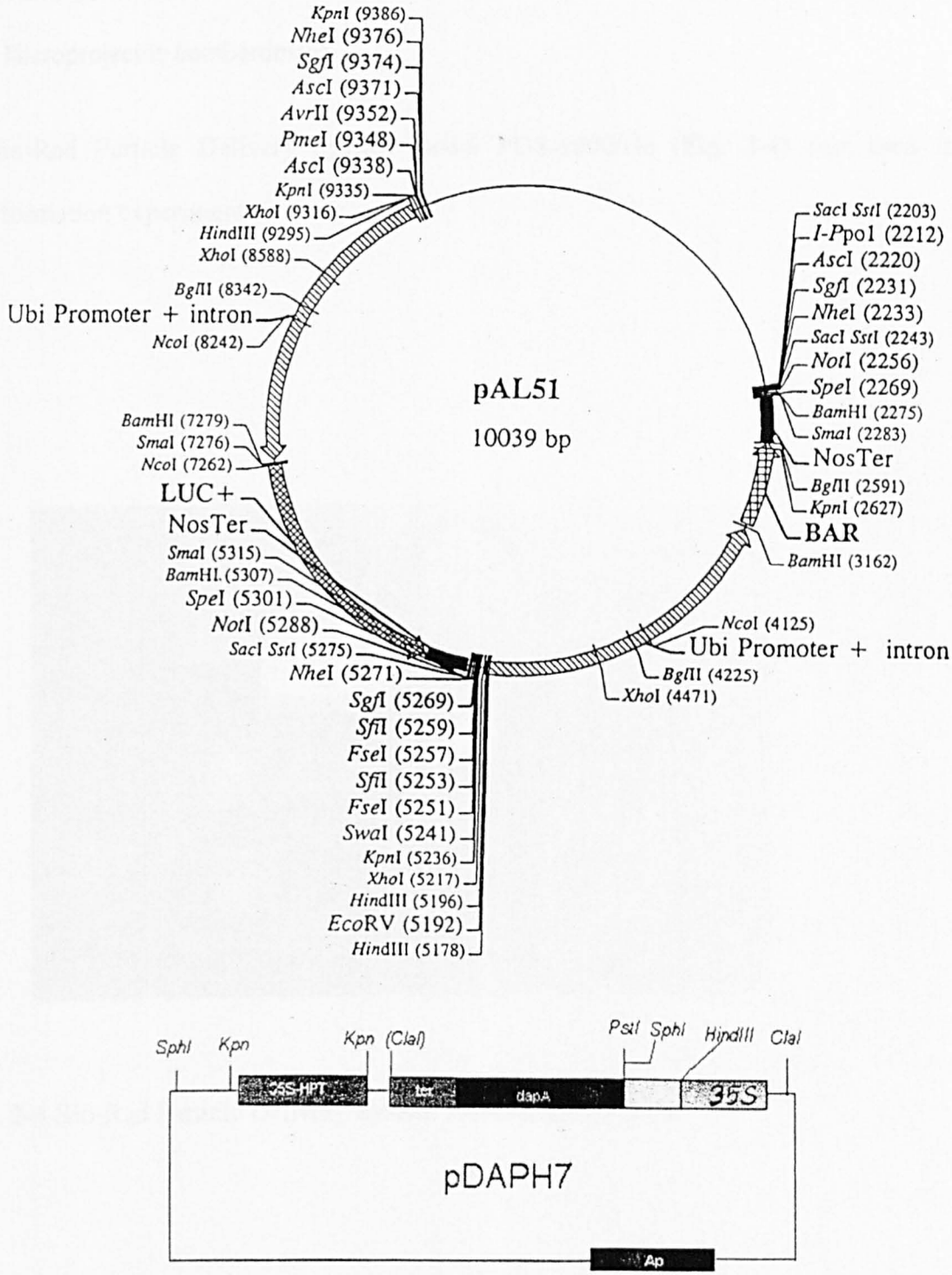


Fig. 2-3 Plasmid constructs used in barley transformation.

2.4 Transformation

2.4.1 Microprojectile bombardment

A Bio-Rad Particle Delivery System model PDS-1000/He (**Fig. 2-4**) was used for transformation experiments.

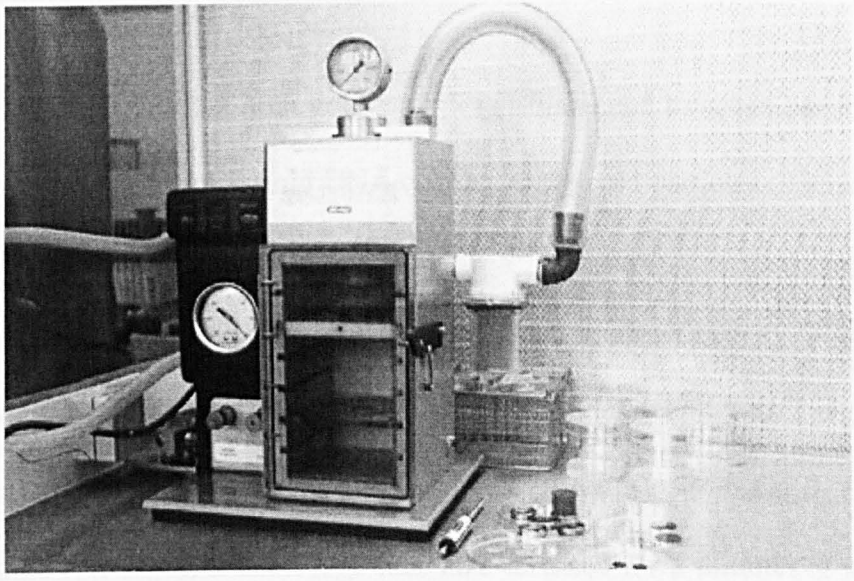


Fig . 2-4 Bio-Rad Particle Delivery System model PDS-1000/He.

Bombardment parameters

For wheat transformation, the following parameters were selected: the distance between the rupture disc and the macrocarrier was 1.5 cm; between the macrocarrier and the stopping plate was 1cm; between the stopping plate and the target tissues was 8 cm. The rupture pressure was 900 psi or 1100 psi, and the partial vacuum was 25-27 inches of Hg.

For barley transformation, Petri dishes with immature embryos were placed in the second grid (about 6cm below the macrocarrier); gas pressure was1100psi and partial vacuum was 27 inches of Hg.

Particles and plasmid DNA coating

In wheat transformation, both gold particles (1.0μm) and tungsten particles (1.1μm) from Bio-Rad were used for plasmid DNA coating. Only gold particles (0.95μm diameter) were used in barley transformation.

DNA-coated gold particles were prepared as described by Becker *et al.* (1994). Prewashed 50 μl aliquots of gold or tungsten particles (contained 2 mg gold or tungsten particles) were mixed with 5 μl plasmid DNA (1 μg/μl), 50 μl $CaCl_2$ (2.5 M) and 20 μl spermidine (0.1 M) in a microfuge tube and vortexed for 2 minutes at room temperature. The mixture was centrifuged in a microfuge for 2 minutes at 10,000 g and the supernatant was removed and discarded. The particles were washed in 250 μl ethanol and re-suspended in 120-200 μl ethanol. For bombardment, 5-10 μl of the DNA-particle suspension was spread on to the surface of the macrocarrier.

For co-transformation of barley, 3μl pDAPH7 (0.7μg/μl) and 2μl pAL51 (1.0μg/μl) were used in one DNA-gold coating and finally dissolved in 150 μl ethanol. 5 μl DNA-gold solution was spread evenly onto the centre of each microcarrier (about 30 microcarriers). Only the macrocarriers with thin layers of well spread particles were used.

2.4.2 *Agrobacterium* strains and co-cultivation with wheat tissues

The binary T-DNA vector pBECKS.red was introduced into two *A. tumefaciens* strains, EHA101 (a 'supervirulent' strain carring a disarmed version of pTiBo542) (Hood *et al.*, 1986) and LBA4404 (Hoekema *et al.*, 1983), by electroporation (McCormac *et al.*, 1998b). Cultures of *A. tumefaciens* were grown in liquid AB medium (Chilton *et al.*, 1974) supplemented with spectinomycin at 28°C in an orbital shaker at 110 rpm for 24-72 hours. The *A. tumefaciens* cell density was adjusted to OD_{600} 0.5 with AB medium immediately prior to co-cultivation, and acetosyringone (AS) was added to the bacterial suspensions (final concentration 100μM). Wheat cultures were immersed in the bacterial suspensions for 20 minutes and then transferred, without rinsing, to fresh MS-AS medium (containing 100μM AS) for co-cultivation in the dark at 25°C for 3 days. Afterwards the cultures were transferred to MS-I medium (containing 500mg/l cefotaxime) in order to inhibit further bacterial growth.

2.5 Selection

2.5.1 PPT selection of wheat

Wheat cultures bombarded with plasmid pDB1 or pAHC25 were either transferred to MSb-PPT medium for early selection and then transferred to fresh PPT5 medium every 2-4 weeks; or transferred to MS-b first and selected on fresh PPT5 medium every 2-4 weeks.

2.5.2 Bialaphos selection of barley

The day after bombardment, the embryos were transferred to BCI5B selection medium which contains 5 mg/l bialaphos, 20 embryos per 9cm dish. Embryos were transferred to fresh selection medium every two weeks. Starting from the second selection (on BCI5B),

the callus from each embryo was broken into small pieces. Only compact, healthy callus was transferred and the origin was recorded. A 1-2 cm gap was left between adjacent callus pieces. Callus was selected on DBC3B medium for 2 weeks before transfer to FWG1B medium for another two rounds of selection. Healthy plantlets were transferred to glass tubes containing plant growth regulator-free BCI1B medium for rooting. Two to three weeks later, green plantlets with healthy roots on BCI1B medium were transferred to soil. Leaf samples were collected for PCR to test for the presence of the *bar* gene. One to two weeks later, Challenge leaf painting was carried out to check *bar* gene expression.

2.6 Detection of gene expression

2.6.1 Detection of *C1/Lc* and *gfp* expression by stereo and epi-fluorescence microscopy

Cultures were checked regularly (from as early as 8 hours to one month after transformation) under a stereo dissecting microscope (Kyowa SDZ-P) for foreign gene expression during the process of regeneration. Cells transformed with *C1/Lc* genes were visualised as red or purple cells (bombarded with pBECKS.red).

For the materials transferred with pBECKS.sgfp-S65T, GFP was visualized using a Nikon epi-fluorescence microscope equipped with an epifluorescence filter set B-3A (excitation filter 420-490 nm, barrier filter 520 nm, dichroic mirror 510 nm). The light source was provided by a 100 W high-pressure mercury bulb. Photographs were taken using Kodak Ektachrome Tungsten 64T colour reversal film.

2.6.2 Detection of *gusA* expression by the histochemical GUS assay

GUS activity was assessed histochemically using 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) (Jefferson *et al.*, 1987). The GUS assay solution was made by dissolving X-GLUC in a small amount (20-60μl) of dimethyl formamide and then adding to phosphate buffer (100 mM $NaPO_4$ pH 7.0) containing 10 mM Na_2EDTA, 0.1% (v/v) Triton X-100,

0.1 mM $K_4Fe(CN)_6.3H_2O$, 0.1 mM $K_3Fe(CN)_6$, and 20% (v/v) Methanol. The final concentration of X-GLUC was 0.5mg/ml.

For transient gene expression, the GUS assay was normally carried out two days after bombardment. Plant tissues (immature embryos or calli) were immersed in GUS assay solution and incubated for 12-24 h in a 37 °C oven. The plant tissues were fixed by replacing the staining solution with 70% ethanol. GUS activity was checked under a stereo microscope.

2.6.3 Detection of *luc* expression by luminography

Transient *luc* expression was tested 2 to 4 days after bombardment. Under sterile conditions, about 50 µl filter sterilized luciferin solution (10µM) was add onto each immature embryo and luciferase activity (Ow *et al.*, 1986) was visualised using a Berthold LUMAT LB9501 luminometer. In a similar way, leaves and roots from regenerated plants, and also compact but not regenerable callus which survived after 4-5 rounds of bialaphos selection, were tested for stable *luc* gene expression. For leaf tissue, cut into small pieces (about 5-10mm), a few drops of 10µM luciferin solution with Tween 20 was used to wet the leaf surface.

2.6.4 Detection of *bar* expression by herbicide testing

Regenerated wheat plants after PPT selection were transferred to soil and tested for resistance to the glufosinate based herbicide Challenge® (containing the active ingredient PPT). Four concentrations of herbicide solution, 1%, 0.5%, 0.1% and 0.05%, were applied to leaves of wheat plants that were grown in a greenhouse. The herbicide was diluted in 0.1% Tween 20 just before use. Two marks were made in the middle of green leaves, about 5 cm apart. The herbicide was applied between the two marks with a paintbrush.

The leaves were checked after herbicide painting and the bleaching effect was recorded one week later.

For putative transgenic barley plants, 1% Challenge was used for leaf painting. At least two young leaves from each plant were tested for resistance to herbicide by marking a 3cm area of each leaf and painting this area with a 1% solution of herbicide. One week after painting, plants were scored for herbicide resistance. Resistant plants showed very little effect of the herbicide whereas sensitive plants were bleached and died from the painted area to the leaf tip.

2.7 Plasmid and plant DNA preparation

2.7.1 Plasmid DNA isolation

Plasmid DNA was isolated from overnight cultures of *E. coli* using standard alkaline-lysis procedures (Sambrook *et al.*, 1989). After precipitation in ethanol, the DNA was re-suspended to a final concentration of 1 μg/μl in sterile distilled water.

2.7.2 Wheat plant DNA preparation

Wheat plant genomic DNA was isolated according to the standard laboratory protocol. The following stock solutions were prepared: DNA extraction buffer (containing 0.35M Sorbitol, 0.1M Tris, and 5.0mM EDTA), nuclei lysis buffer (containing 0.2M Tris, 0.05M EDTA, 2M NaCl, and 2% CTAB), and 5% N-Lauroylsarcosine. DNA extraction solution was made by mixing the three stock solutions at a ratio of 5:5:2 (in volume), and adding sodium bisulfite to a concentration of 0.3%(w/v) before using.

50-100mg young leaf tissue was ground and incubated with 600μl DNA extraction solution in a 1.5ml tube at 65°C for 60 minutes, followed by 500μl chloroform/isoamyl (24:1) extraction. The mixture was vortexed, then centrifuged at 10,000 rpm for 5 mins. The aqueous phase (about 0.4-0.5 ml) was pipetted off into a new tube and 2/3 to 1 time of

volume of cold isopropanol was added for DNA precipitation. The mixture was centrifuged at 10,000 rpm for 5 mins then the isopropanol was poured off. The pellet was washed with 70% ethanol, centrifuged for 5 mins before the ethanol was discarded and the pellet air dried for 1 hour. The DNA was resuspended in 50µl TE or water and stored at -20°C.

2.7.3 Barley plant genomic DNA preparation for PCR

Genomic DNA for PCR analysis was extracted using the procedure of Edwards *et al.* (1991). The DNA extraction buffer contained 200 mM Tris HCl pH7.5, 250mM NaCl, 25 mM EDTA and 0.5% SDS. Fresh extraction buffer was made from autoclaved stock solutions. Fresh leaf samples from young plantlets were collected in Eppendorf tubes and macerated with liquid nitrogen using disposable grinders. 400 µl of extraction buffer was added and the sample was vortexed for 5 seconds. The extracts were centrifuged at 13,000 rpm for 1 minute and 300 µl of the supernatant were transferred to a fresh Eppendorf tube. This supernatant was mixed with 300 µl isopropanol and left at room temperature for 2 minutes. Following centrifugation at 13,000 rpm for 5 minutes, the pellet was dried in a lamina flow hood and dissolved in 100 µl 1×TE.

2.7.4 Barley plant genomic DNA preparation for Southern analysis

Barley genomic DNA was isolated from leaf tissue using a modified CTAB (Murray and Thompson, 1980) protocol. Leaf materials of herbicide resistant, PCR-positive plants or non-transformed control plants were ground to a fine powder in liquid nitrogen and then mixed with 10ml of CTAB buffer (25g Sorbitol, 10g N-Lauroylsarcosine, 16g CTAB, 47g NaCl, 8g EDTA and 12.5g PVPP made up to 1 L in 0.2M Tris) and incubated at 65 °C for 1 to 2 hours with occasional mixing. Two chloroform extractions were carried out and then the DNA was precipitated with one volume of isopropanol. The DNA pellet was

dissolved in 10μmol Tris, RNase A treated and then subjected to one phenol/chloroform extraction before being Ethanol precipitated, dried and redissolved in 10μmol Tris.

2.8 Analysis of plants using the polymerase chain reaction (PCR)

2.8.1 Primers

PCR was performed either for testing putative transformants, or generating gene probes for Southern analysis.

For wheat plants, the presence of the *sgfp* gene was determined by amplification of a 735 bp fragment of the gene using the primer pair 5'-GGA TCC ATG GTG AGC AAG GGC-3' (position on *gfp* 22→42) and 5'-GCG GCC GCT TTA CTT GTA CAG-3' (position on *gfp* 736←756), and the *bar* gene by amplification of a 534 bp fragment of the gene using primers 5'-GAG ACC AGT TGA GAT TAG GCC-3' (position on bar 20→40) and 5'-ATC TGG GTA ACT GGC CTA ACT-3' (position on bar 533←553) (according to Becker *et al.*, 1994).

For barley plants, the presence of the *bar* gene was determined by the amplification of a 421 bp fragment of the gene using the primer pair 5'-GGT CTG CAC CAT CGT CAA CC-3' and 5'-GTC ATG CCA GTT CCC GTG CT-3', the *luc* gene by amplification of a 1.158kb fragment of the gene using primers 5'-GCC GGT GTT GGG CGC GTT-3' and 5'-GCG GGA AGT TCA CCG GCG-3' and the *dapA* gene by amplification of a 892 bp fragment of the gene using primers (DAPA3) 5'-CTA AAC TTT ACA GCA AAC CGG-3' (Tm= 60.2°C) and (DAPA5) 5'-ATG TTC ACG GGA AGT ATT GTC-3' (Tm=60.3°C). The primers were ordered from Genosys Biotechnologies (Europe) Ltd (according to the protocols in Dr Harwood's lab in JIC).

2.8.2 Reaction solution

The wheat PCR reaction mixture contained the following reagents: 5μl 10× Taq (*Thermus aquaticus*) amplification buffer ($MgCl_2$ free), 5μl $MgCl_2$ (25mM), 5μl dNTP (2mM each), 5μl of both primers (2μM), 0.5μl Taq polymerase (Promega), 1μl DNA template (containing 0.1ng plasmid DNA or 3μg plant genomic DNA), plus 23.5μl sterile distilled H_2O to make 50μl of final reaction mixture. Three drops of mineral oil were added to cover the top of the reaction mixture before PCR amplification started.

The barley PCR reaction solution contained the following reagents: 2.5 μl 10× Taq (*Thermus aquaticus*) amplification buffer (with $MgCl_2$), 2.5 μl dNTP's (2mM each), 1 μl of both primers (10 μM), 0.2μl Taq polymerase (Promega), plus 17μl sterile distilled H_2O to make 24μl of PCR mixture. Then 1μl of DNA sample for PCR was added to each tube containing 24 μl of the PCR mix to make the final reaction mixture up to 25 μl. For the positive control, 1μl 1ng/μl or 10 pg/μl plasmid was used; for the blank control, 1μl H_2O was used.

2.8.3 PCR programmes

For wheat

PCR amplification was carried out using DNA engine PTC-200 Peltier Thermal Cycler. Three PCR amplification programmes, Programme I, II and III, were used.

The thermal cycling **Programme I** was 94°C for 3 minutes (denaturation), then 40 cycles of 94°C for 30 seconds (denaturation), 60°C for 60 seconds (primer annealing) and 72°C for 90 seconds (extension). A final extension step for 5 minutes at 72°C was performed prior to cooling to 4°C.

The thermal cycling **Programme II** (for labelling PCR-DIG-Probe) was 95°C for 3 minutes, then 35 cycles of 95°C for 30 seconds, 60°C for 1 minute and 72°C for 2

minutes and 30 seconds. A final extension step for 5 minutes at 72°C was performed prior to cooling to 4°C.

The thermal cycling **Programme III** (for *sgfp* PCR) was 94°C for 3 minutes, then a cycle of 94°C for 45 seconds, 70°C for 60 seconds and 72°C for 90 seconds, followed by 9 similar cycles but reducing annealing temperature by 0.5°C each cycle. Then a further 25 cycles of 94°C for 45 seconds, 68°C for 60 seconds and 72°C for 90 seconds. A final extension step for 5 minutes at 72°C was performed prior to cooling to 4°C.

For barley

Three PCR amplification programmes were used for BAR, DAPA and LUC-PCR.

The thermal cycling BAR-PCR programme (Touchdown PCR conditions for the *bar* gene) was 94°C for 1 minute, then a cycle of 94°C for 45 seconds, 70°C for 45 seconds and 72°C for 45 seconds, followed by 9 similar cycles but reducing the annealing temperature by 0.5°C each cycle. Then a further 25 cycles of 94°C for 45 seconds, 65°C for 45 seconds and 72°C for 45 seconds. A final extension step for 5 minutes at 72°C was performed prior to cooling to 4°C.

The thermal cycling DAPA-PCR programme was 94°C for 3 minutes, then 30 cycles of 94°C for 45 seconds, 60°C for 90 seconds and 72°C for 90 seconds. A final extension step for 5 minutes at 72°C was performed prior to cooling to 4°C.

The thermal cycling LUC-PCR programme was 94°C for 5 minutes, then 35 cycles of 95°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute. A final extension step for 5 minutes at 72°C was performed prior to cooling to 4°C.

2.8.4 PCR labeling of probe DNA (for wheat)

PCR DIG probe Synthesis Kit (Boehringer Mannheim), a non-radioactive labelling kit, was used for generation of probes labelled with DIG-dUTP. The complete PCR mixture contained the following agents: 5μl 10× Taq (*Thermus aquaticus*) amplification buffer (with $MgCl_2$), 5μl 10× DIG-nucleotide mixture, 5μl of both Primers (2μM), 0.75μl (2.6U) Taq polymerase, 1μl (0.1ng/μl) plasmid DNA (pBECKS-sGFP or pDB1). Sterile distilled H_2O was added to a final volume of 50μl. PCR programme II was operated to generate the

PCR-DIG-Probe. PCR products (15µl) were separated by running a mini-gel, and the probes (of the correct size) were purified using the Geneclean II® Kit (Bio 101 Inc., La Jolla, USA).

2.8.5 Visualisation of PCR products

PCR amplifications were checked on 1.2% agarose gels. A mini-gel was made by dissolving 0.6g agarose in 50 ml 1×TAE buffer (using microwaves to facilitate solution), and adding 1µl ethidium bromide (10µg/µl). 10µl of PCR product was used and 2µl tracking dye (0.25% w/v xylene cyanol, 0.25% w/v bromophenol blue and 25% w/v Ficoll Type 400) was added, mixed well and loaded on a gel. 2µg of λ DNA digested with *Eco*RI and *Hind*III was also loaded as a size marker. The gel was run at 65 V for 60-90 mins until the products of PCR amplification were well separated. PCR amplifications were visualized using either an UV trans-illuminator or a Bio-Rad Multi-Analyst™/PC. A 735bp fragment of the *gfp* and a 534bp fragment of the *bar* gene from wheat PCR were used as probes for Southern hybridization. A 421bp fragment of the *bar* gene from barley PCR was used as a probe for the Southern analysis.

2.9 Southern blot hybridization for wheat

2.9.1 Gel electrophoresis

Twenty micrograms of each plant DNA sample were digested with *Hind*III and/or *Eco*RI in 30µl of the manufacturer's buffer over night. Twenty micrograms of digested or undigested plant genomic DNA were separated by electrophoresis in 0.8% (w/v) agarose gels (Sambrook *et al.*, 1989). Plasmids (10-100pg of plasmid used to generated transgenic material) and DNA samples of untransformed plants were used as positive and negative controls.

2.9.2 Southern transfer

The DNA was transferred from the agarose gel to Hybond^{TM}-N^{+} Nylon Transfer membrane (Amersham), or Quantum Yield^{TM} Hybridization membrane (for non-radioactive labeling, Promega) following a protocol described by Amersham. The dry membrane was put in an 80°C oven for 2 hours, for DNA-membrane cross-link.

2.9.3 Southern blot hybridization

Filters were hybridized with a PCR-DIG-labeled 500bp *bar* or 700bp *gfp* probe, according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Two hybridization buffers were used: standard hybridization buffer (without formamide) and standard hybridization buffer with formamide. The first buffer contained 5×SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 1% blocking reagent, hybridization temperature was 68°C. The second buffer contained 5×SSC, 50% deionized formamide, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 2% blocking reagent, hybridization temperature was 42°C. The hybridization solution was made by adding 10µl denatured DIG-PCR probe (*gfp* or *bar*) (purified from 15µl PCR product) to 10ml hybridization buffer.

The hybridization oven (Hybond) was set at 68°C or 42°C, according to the hybridization buffer applied. 30ml of hybridization buffer were used to pre-hybridize the membrane for 2-4 hours. Then the membrane was hybridized with the hybridization solution overnight (18 hours).

Post-hybridization, the membranes were washed 2×5 min in ample 2×SSC, 0.1% SDS at room temperature, and 2×15 min in 0.1×SSC, 0.1% SDS at 68°C under constant agitation.

2.9.4 Probe detection and autophotography

Hybridized probe DNA was detected using a chemiluminescent detection system CDP-star™ (Boehringer Mannheim, Mannheim, Germany). Hybridization signals were visualized on Hyperfilm™ High performance chemiluminescence film (Amersham) by exposing to the hybridized membrane for 5min to 3 hours at room temperature.

2.10 Southern blot hybridization for Barley

The barley genomic DNA (10μg/sample) was digested with *Bgl*II, electrophoresed in 0.8% agarose gel and transferred to a Hybond N^+ membrane (Amersham) according to Southern (1975). The membrane was hybridized with a ^{32}P labelled 421 bp fragment of the *bar* gene. The bar probe was purified from the PCR product using a QIA quick Gel Extraction Kit.

Chapter Three

PLANT REGENERATION

3.1 Introduction of somatic embryogenesis

Whole plants can be obtained from cultured plant cells and tissues either by organogenesis, via shoot and root formation respectively, or via somatic embryogenesis, embryoids formed from somatic cells with characteristics similar to the zygotic embryos. The embryoids induced from cultured plant tissues often develop a region equivalent to the suspensor of zygotic embryos and come to have both a shoot and a root pole to distinguish them from zygotic or seed embryos (George EF, 1993).

The early stages of embryogenesis in maize are illustrated in **Fig. 3-1** (from Meinke, 1991) and stages of somatic embryogenesis in monocotyledons are illustrated in **Fig. 3-2** (from George, 1993). In general the two processes are morphologically similar from the globular stage onwards. There may be differences in cell number or degree of expansion at particular stages, and somatic embryoids are frequently observed to have more than the normal number of cotyledons (Williams and Maheswaran, 1986).

Somatic embryogenesis occurs in cell or organ cultures of a broad range of species. Williams and Maheswaran (1986) reviewed common features of somatic embryogenesis *in vitro*. Somatic embryos (embryoids) originate from either single-cells or multiple-cell clusters. **Fig. 3-3** illustrates the initiation of the embryoids in somatic embryogenesis (from Williams and Maheswaran, 1986). In general, a multicellular origin appears to produce embryoids fused with the parent tissue over a broad area of the root pole or axis region, whereas a unicellular origin is more likely to give embryoids attached by a narrower suspensor-like organ. Haccius (1978) defined a non-zygotic embryo as 'a new individual arising from a single cell and having no vascular connection with maternal tissues'. Both multicellular budding and single-cell initiation of embryoids are observed from the same type of proembryonal complex (reviewed in Williams and Maheswaran, 1986).

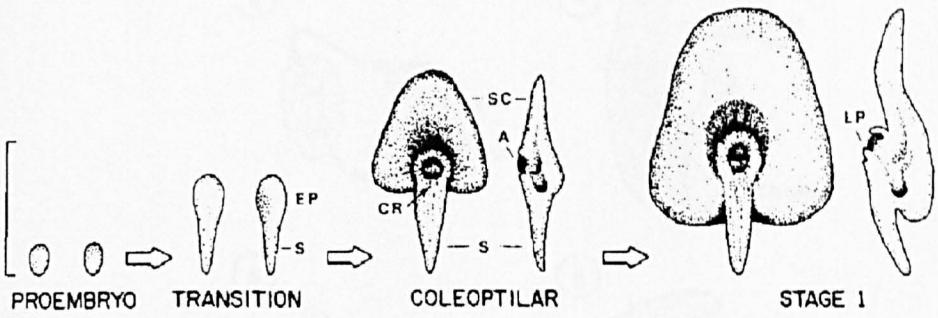


Fig. 3-1 Early stages of embryogenesis in Maize. Each stage is represented by a face view (left) and a longitudinal section (right) with the embryo face at left. The transition embryo contains an embryo proper (EP) and suspensor (S). The scutellum (SC), shoot apex (A), suspensor (S), and coleopillar ring (CR) are visible at the coleopillar stage. The first leaf primordium (LP) appears at the base of the shoot apex at stage 1. Bar = 0.5 mm. Figure from Meinke DW (1991).

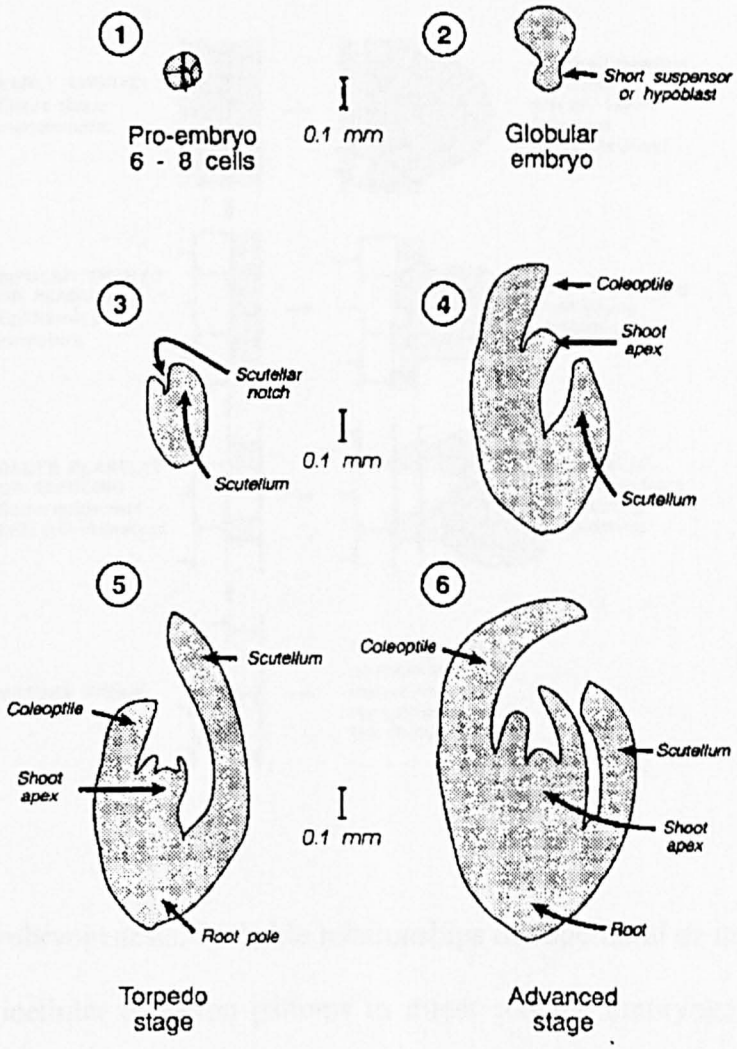


Fig. 3-2 Stages of somatic embryogenesis in monocotyledons. Figure from George EF (1993).

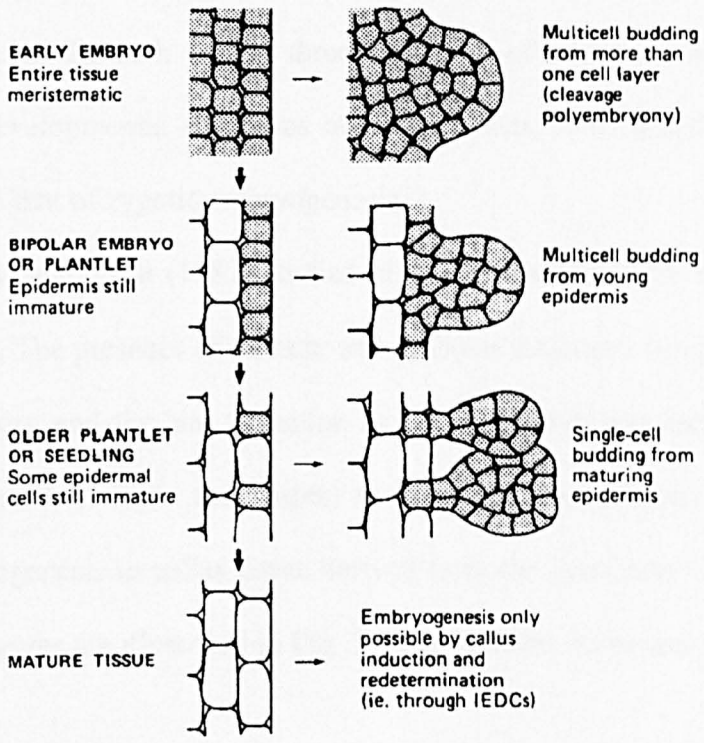


Fig. 3-3 Somatic embryogenesis. Probable relationships of superficial or multy-layer, and single-cell or multicellular initiation patterns in direct somatic embryogenesis. Shading indicates pre-embryogenic determined cells. IEDCs, induced embryogenic determined cells. Figure from Williams EG and Maheswaran **G** (1986).

Magnusson I and Bornman CH (1985) investigated somatic embryogenesis from scutellar tissues of immature zygotic embryos of *Triticum aestivum.* Somatic embryos were observed as early as six days after subculturing immature embryos of *Triticum aestivum in vitro* on 2,4-D containing nutrient media. Embryo formation followed three pathways, each involving one of the scutellum's three basic tissue systems: dermal, ground and vascular. In each of the three pathways of embryoid formation in their investigation, a developmental stage was observed which, in its morphology, showed a striking analogy to that of zygotic embryogenesis.

Ozias-Alins and Vasil (1982) studied plant regeneration from cultured immature embryos of wheat. The presence of nodular and globular structures (original paper Figs 6-8, 10 in their paper), and the late formation of a lateral notch and leafy structures from these nodules (Figs. 9, 11-22 in their paper) were presented as evidence for the initiation of somatic embryogenesis in callus tissue derived from the scutellum of immature wheat embryos. These figures are illustrated in **Fig. 3-4** (from Ozias-Alins and Vasil, 1982).

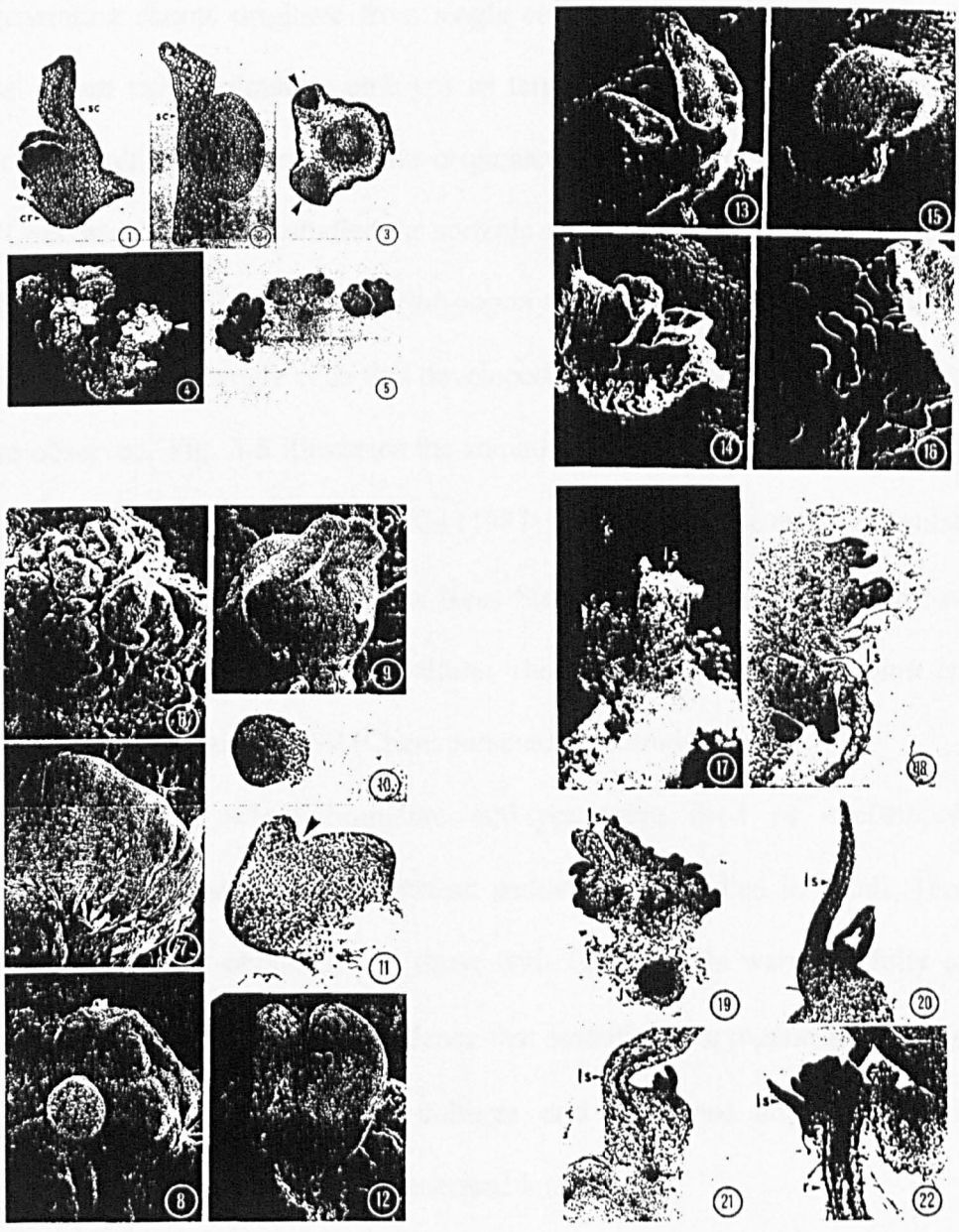


Fig. 3-4 Evidence for somatic embryogenesis in callus tissue derived from the scutellum of immature wheat embryos. Figure from Ozias-Akins P and Vasil IK (1982).

Although it is believed that plant regeneration from the embryo's scutellum involves somatic embryogenesis (Ozias-Akins and Vasil, 1982), it is not known whether the regenerating shoots originate from single cells within the scutellum of the original explants. When using immature embryos as targets for transformation, it is not known whether the resulting transformed plants originated from single cells or groups of cells.

Chen and Xia (1986) studied the somatic embryo formation and plant regeneration from cultured young inflorescence of *Polypogon fugax* Nees Steud. They used histological sectioning and located single cells that developed into a full SE; there was also suspensor structure observed. **Fig. 3-5** illustrates the somatic embryogenesis in the *Polypogon* grass (from Chen and Xia, 1986). Chen and Xia (1987) reported somatic embryo formation from protoplast cultures of *Polypogon fugax* Nees Steud. They showed SE from low-density protoplast (single cell) hanging drop culture. They also studied the protoplast cultures of wheat, but failed to obtain any SE (Chen, personal communication).

In this study, wheat immature embryos were used as explants for plant regeneration. The wheat plant regeneration pathway was studied in detail. Transformed somatic embryos were obtained, and those with GFP signals were carefully monitored until maturity. The results provide evidence that somatic embryogenesis from single cells occurred in wheat immature embryo cultures, and that those single cells in immature embryos could be transformed and regenerated into plants.

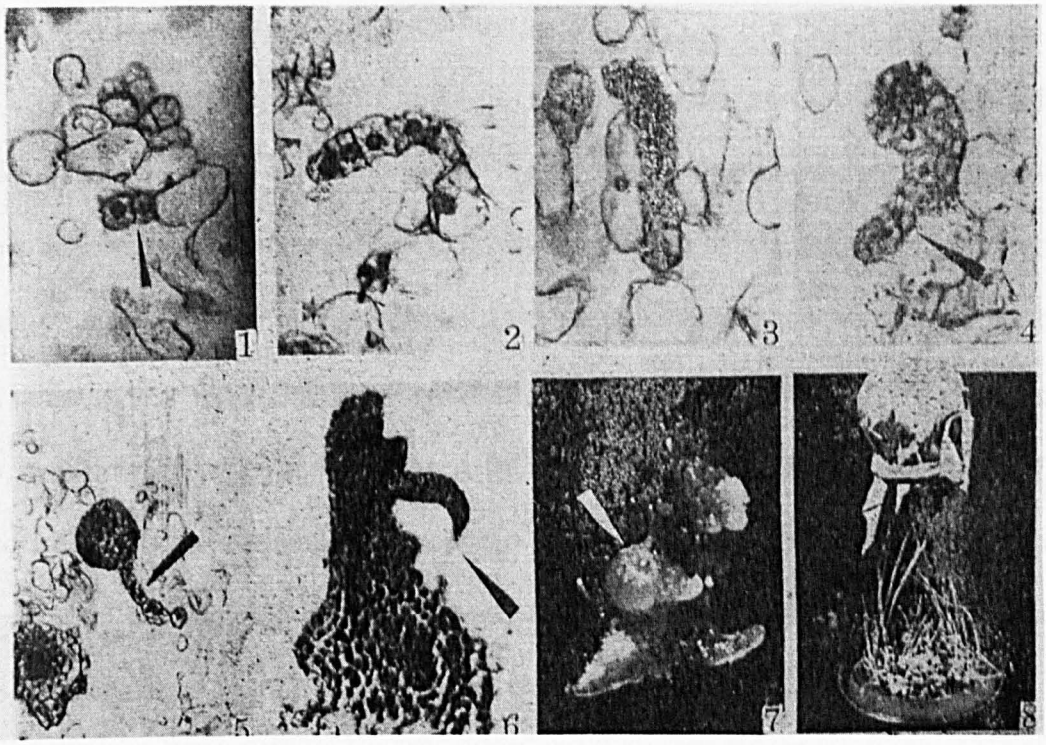


Fig. 3-5 Somatic embryogenesis in cultured young inflorescence of *Polypogon fugax* Nees Steud. Figure from Chen and Xia (1986).

3.2 Somatic embryogenesis

3.2.1 Regeneration of wheat plants from immature embryo cultures

Immature embryos of wheat were used as explants for plant regeneration. **Fig. 3-6** illustrates the process of plant regeneration from an immature embryo (Rascal, a commercial U.K. wheat variety, in this case).

Freshly isolated immature embryos (**Fig. 3-6a**) were inoculated on callus induction medium MS-b which contained 2mg/l 2,4-D. About five days after initiation of the immature embryo cultures, proliferating callus emerged as a ridge around the periphery (mainly) and protrusion on the surface of the scutella. About two weeks after the culture was initiated, somatic embryos became distinguishable (**Fig. 3-6b**). In the light, green leaf-like structures emerged and developed from the embryonic cluster (**Fig. 3-6c**). Two to three weeks after initial immature embryo culture, the tissues were transferred to plant regeneration medium MS-r that contained 0.5mg/l NAA and 0.5mg/l KT. The somatic embryos developed further, forming green shoots and roots (**Fig. 3-6d**).

Two types of callus were produced by the immature embryo cultures: white, watery callus (like the central tissue in **Fig. 3-6b**) and yellow, compact callus (like the peripheral tissue in **Fig. 3-6b**). The white, watery callus grew fast and was friable, but not regenerable. The compact callus usually had a smooth surface and was regenerable. Plantlets were regenerated only from compact callus. Sometimes, however, compact callus seemed to have arisen from the underside of the friable, watery callus, and regenerated plantlets. This was probably due to the fast swollen callus covering the regenerable callus at an early stage of callus induction.

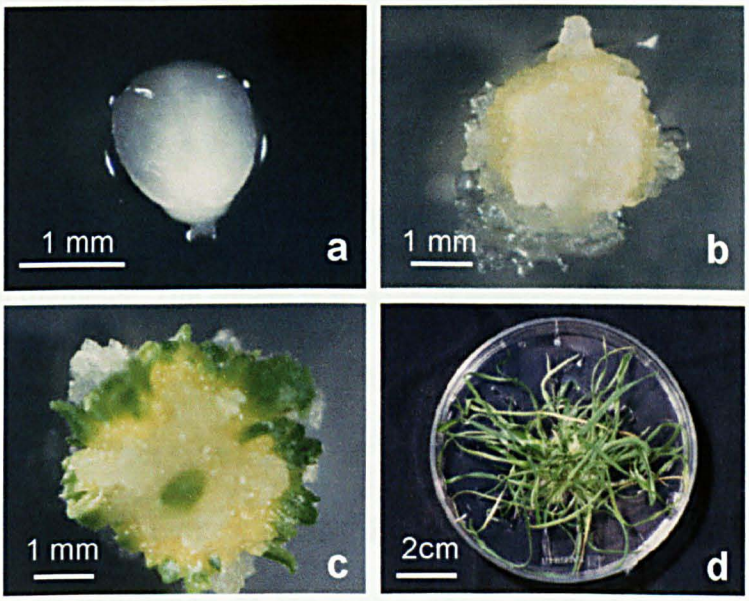


Fig. 3-6 Regeneration of wheat plantlets from an immature embryo. **(a)** A freshly isolated immature embryo. **(b)** Embryogenic callus (yellowish compact callus) and non-embryogenic callus (transparent watery callus) emerging from the edges of the immature embryo. **(c)** Green leaf-like structures developed from somatic embryos. **(d)** Shoots and roots developed from the leaf-like structures.

3.2.2 Somatic embryogenesis: formation and appearance of somatic embryos

The formation and appearance of some somatic embryos is shown in **Fig. 3-7**. Some somatic embryos showed transgene expression after biolistic bombardment with *C1/Lc* (**Fig. 3-7b**) or *gusA* **(Fig. 3-7f)**.

Typical somatic embryos were observed as identifiable structures at the globular embryo stage. **Fig. 3-7a** shows a globular embryo (in the centre of the picture) among compact callus. It was a yellowish sphere, about 200μm in diameter; the scutella cell is about 15-25μm in diameter. The sphere is in contact with the parental tissue at the base. A 'ruby' somatic embryo at this stage (about 160μm in diameter) was also observed (**Fig. 3-7b**). It was obtained by direct gene transfer of the plasmid pBECKS.red (containing *C1/Lc* reporter) to the scutellum of wheat (shown here from immature embryos of the spring wheat 'Rascal'). The expression of the *C1/Lc* genes resulted in the accumulation of anthocyanin. The whole embryo appeared 'ruby' and the colour was associated exclusively with this embryo. The short suspensor was visible at the root pole. This 'ruby' somatic embryo had probably been developed from a single transformed cell.

The development of somatic embryos was not synchronous. Somatic embryos at different developmental stages were found in the culture originating from a single immature embryo, as shown in **Fig. 3-7c**. Both typical embryo structures (SE2 & SE3) and some embryos with scutella elongated into green leaf-like structures (SE1) are shown. In the centre of the picture (**Fig. 3-7c)** the embryos (SE2 & SE3) are yellowish, about 0.3mm in diameter. They have scutella notches, like zygotic embryos at their early developmental stage. More advanced somatic embryos have their scutella elongated to green leaf-like structures (SE1). These somatic embryos cluster together on compact tissue, and the individuals are not easily separated from each other (**Fig. 3-7d**, a top view of **Fig. 3-7c**).

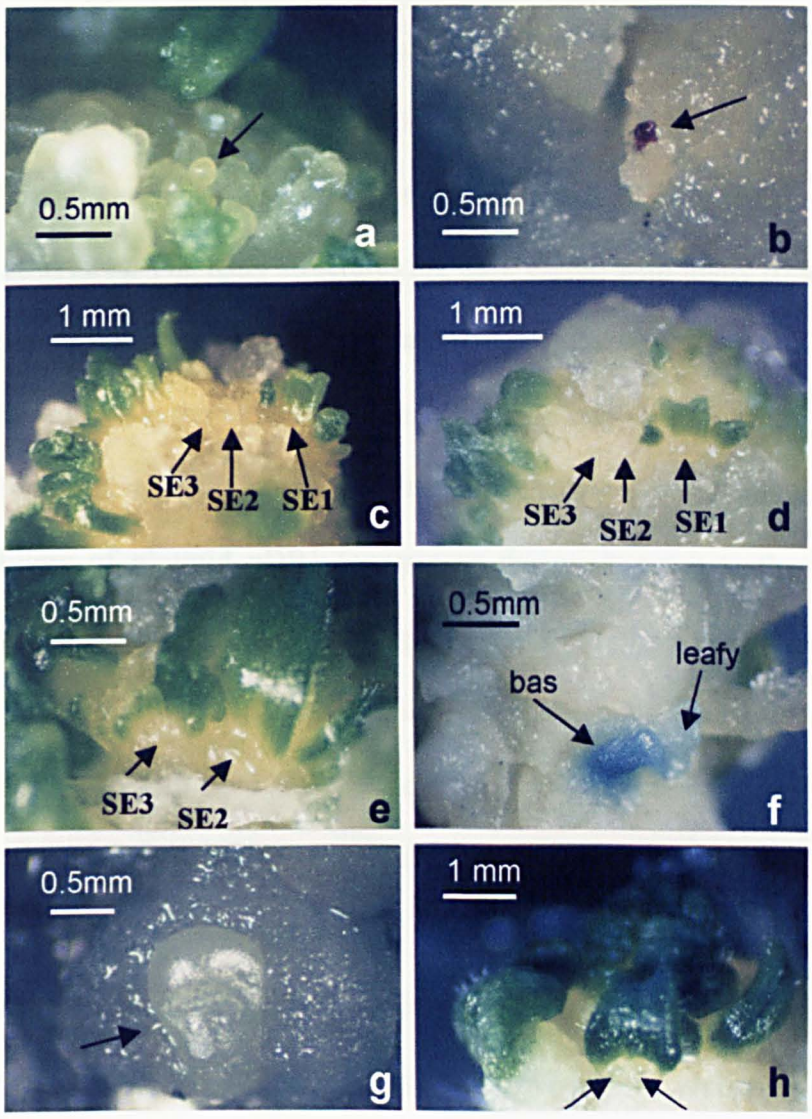


Fig. 3-7 Somatic embryogenesis. (**a**) A globular somatic embryo in the centre of the picture. (**b**) A 'ruby' somatic embryo expressing the *C1/Lc* reporter genes. (**c**) Yellowish somatic embryos in the centre of the picture. More advanced somatic embryos have their scutella elongated to green leaf-like structures. (**d**) A top view of **c**. (**e**) Further development of the yellowish somatic embryos in **c**. (**f**) A 'blue' embryo expressing GUS activity. (**g**) A zygotic-like somatic embryo. (**h**) Two shoot primordia under one leafy structure. See text for details.

The first appearance of green tissue on the immature embryo culture was usually not associated with real leaves, but in structures derived from elongated scutella of somatic embryos. The zygotic embryo-like embryoids SE2 & SE3 shown in the centre part of **Fig. 3-7c** have developed, in one week, into the structures shown in **Fig. 3-7e**, similar to SE1 at **Fig. 3-7c** a week earlier. The scutella tissues elongated into leaf-like structures, or so called leafy structures. The leafy structures are dark green, with many trichomes. Shoot primordia have formed at the base of green leafy structures, about 0.5-0.7mm in diameter. A 'blue' somatic embryo at the same developmental stage was also found (**Fig. 3-7f**). This 'blue' somatic embryo, about 0.5mm in diameter at the leafy base, was obtained after direct gene delivery of plasmid pDB1 (containing the *gusA* gene) to the scutellum of wheat (shown here for immature embryos of spring wheat 'Scamp') and stained with GUS assay solution. The whole embryo was stained blue, albeit the blue colour was much lighter in the leafy structure than that in the base. The blue staining was exclusively associated with the discrete embryo structure. Therefore, this blue embryo should have originated from a single transformed cell. It would have developed into a transformed plant if it had not been killed during the destructive GUS assay.

Somatic embryos at the torpedo stage (like that of zygotic embryos) were seldom observed. **Fig. 3-7g** shows such a somatic embryo, which is similar to an immature zygotic embryo. This kind of somatic embryo normally suffered from arrested development. The shoots were usually generated from somatic embryos whose scutella had already elongated and changed into green leafy structures. The shoot and leaf primordia were formed at the bases of the leafy structures. Commonly more than one shoot primordium was present under each leafy structure. **Fig. 3-7h** shows two shoot primordia (each primordium was about 500μm in diameter) under one leafy structure. These multiple shoot primordia could readily germinate. It was not clear whether these multiple shoot primordia had the same

origin, or were derived from individual somatic embryos but fused together on the scutellum. A 'half-blue' leafy structure obtained by transformation (**Fig. 4-3c**) suggested that the leafy structure with multiple shoot primordia may have resulted from fused scutella (see Chapter 4, Section 4.3.1).

3.2.3 Precocious germination of somatic embryos: development of leaves and roots

The precocious germination of somatic embryos resulted in plantlet formation. It was the major plant regeneration pathway in immature embryo cultures of wheat. The precocious germination of somatic embryos was usually associated with the development of green leafy structures. Shoot primordia were located at the base of leafy structures. Primordia of axillary buds could form at the same time as leaf primordia were developing. Therefore, "micro-tillering" occurred and these structures developed during the germination of the somatic embryos. **Fig. 3-8a, b & c** show the development of leaves from yellowish shoot primordia of somatic embryos. Usually more than one shoot meristem was observed under each leafy structure (**Fig. 3-8a**). Primordia of axillary buds sometimes emerged at the bases of the green leafy structures during the development of true leaves (**Fig. 3-8b & c**). Not all shoot meristems developed into shoots. Some of the shoot primordia (meristems) failed to form any leaves (**Fig. 3-8d**). This was probably due to nutrient deficiency, for there were overcrowded meristems clustering together. The roots of somatic embryos were developed from the root poles, opposite the newly formed shoots (**Fig. 3-8e**). Root hairs were developed (**Fig. 3-8f**).

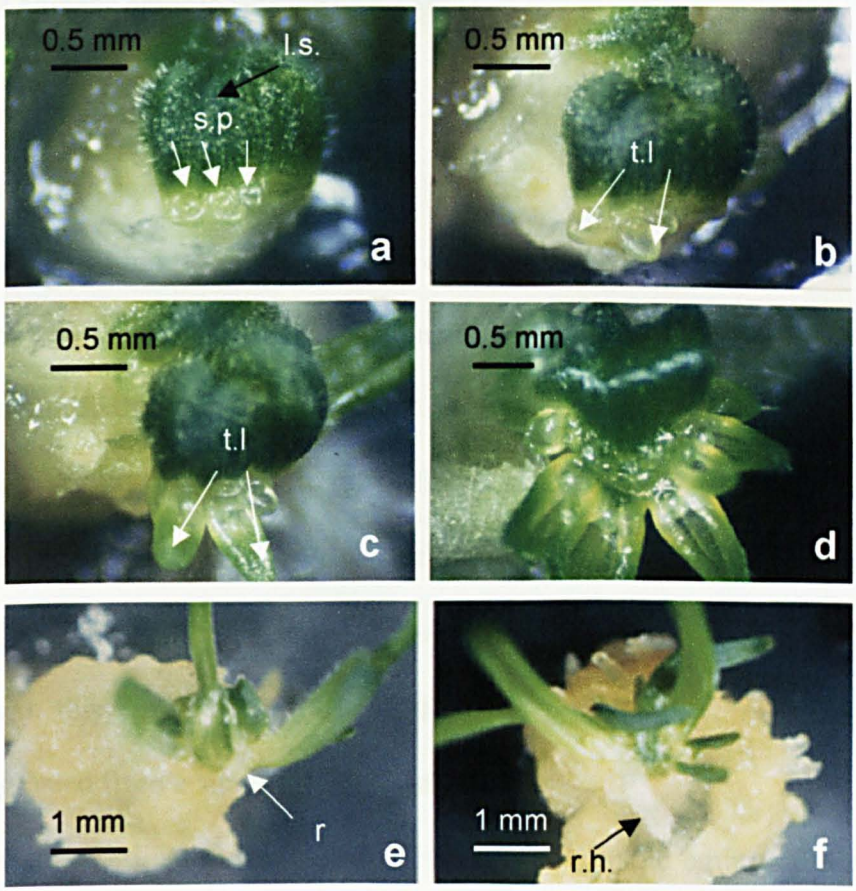


Fig. 3-8 Precocious germination of a somatic embryo: development of leaf and root. **(a)** Three shoot primordia (s.p.) are located, side-by-side, at the base of the green leafy structure (l.s.). Each shoot meristem is about 0.3mm in diameter. **(b)** The same tissue as in **a**, five days later. The first true leaves (t.l.) are developing from the leaf primordia. Meanwhile, more shoot meristems (or axillary buds) are forming at the base of the green leafy structure. **(c)** The first true leaves (t.l.) become green. There are two shoot meristems at this stage, between the green leafy structure and each of the two green leaves (at the left of the picture). The meristems at the outer side have been covered by the second leaves. This picture was taken ten days later than **a**. **(d)** Two rows of shoot meristems under the leafy structure. The row of shoot primordia near to the leafy structure failed to form leaf primordia. **(e)** A root (r) has emerged from the root pole, opposite to the shoot. **(f)** Root hairs (r.h.) have developed.

3.2.4 Further evidence of the single cell origin of somatic embryos: the development of a plantlet from a somatic embryo labelled with *sgfp*

The development of a somatic embryo was followed from the early globular stage to plantlet formation. This somatic embryo was 'labelled' with *sgfp* (the green fluorescence protein gene). It was produced by direct gene delivery of the plasmid pBECKS.sgfp-S65T (containing the *gfp* gene) to scutella tissue one day after the initial wheat (Kedong 58, in this case) immature embryo culture. Twelve days after gene delivery (13 days after the initial immature embryo culture), a green-fluorescing somatic embryo was identified among the nodule structures (**Fig. 3-9a, b & c**). It was at the globular stage, about 400μm in diameter. The entire somatic embryo fluoresced, and the fluorescence was associated exclusively with this embryo (**Fig. 3-9a & b**). This green-fluorescing somatic embryo must have originated from a single transformed cell.

A shoot primordium emerged at the base of the somatic embryo three days later (**Fig. 3-9d**). The top of the embryo extended to a leafy structure. The flanks of the leafy structure's base were expanding and, at this stage, they partially covered the shoot primordium, like a coleoptile. Later, a shoot and roots developed from the somatic embryo (**Fig. 3-9e & f**). The coleoptile-like structure also expanded and gradually covered the whole shoot base. The process of plantlet regeneration from the initial immature embryo culture took 40 days. The plantlet was later transferred to soil. It grew healthily in the greenhouse and set seeds.

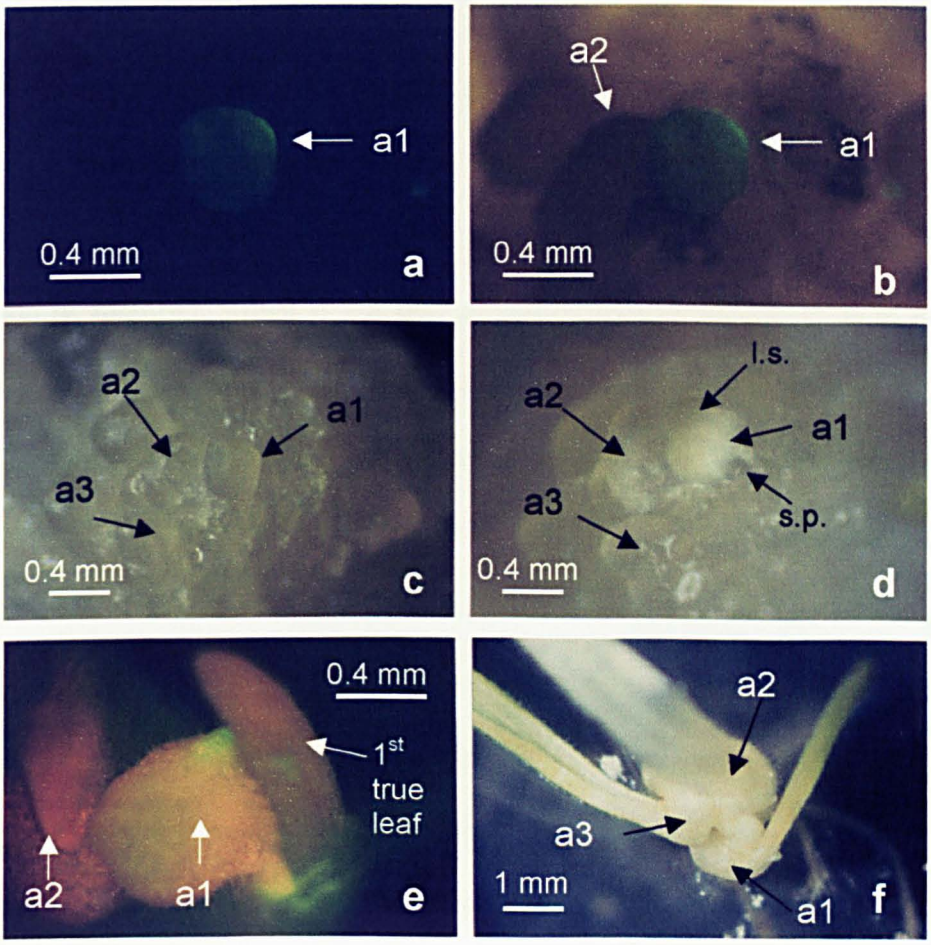


Fig. 3-9 The development of somatic embryos. One somatic embryo (coded as 'a1') expressed GFP. See next page for the full legend.

Fig. 3-9 (continued) Wheat immature embryos (cultivar Kedong 58) were bombarded with the plasmid pBECKS.sgfp-S65T carrying an *sgfp (S65T)* gene, one day after the initial culture. The green fluorescence was traced during callus development using a fluorescence microscope under blue light, with a Nikon B-3A filter set (420-490 nm). (**a**) A somatic embryo 12 days after gene delivery. The whole somatic embryo (a1) emits mild green fluorescence, with a very bright area at one edge. (**b**) The same tissue as in **a**, with additional normal lighting. The fluorescing embryo (a1) is distinguishable from its neighbour (a2, in shadow), which is not fluorescing. (**c**) The same tissue as in **a** and **b**, viewed under normal (tungsten) light using a stereo dissection microscope. The GFP 'labelled' somatic embryo (a1) and its surrounding tissue (including somatic embryos a2 & a3) are shown. The 'green' somatic embryo (a1) is in the middle of the picture, about 400μm in diameter. (**d**) A shoot primordium (s.p.) has emerged at the base of the somatic embryo (a1), and the top of the somatic embryo becomes green leafy structure (l.s.). The flanks of the leafy base are expanding and partially cover the shoot primordium, like a coleoptile. The photograph was taken 16 days after the initial immature embryo culture, three days later than **c**. (**e**) Germination of the somatic embryos. The first true leaves (t.l.) have developed from the shoot primordia. Green fluorescence remains visible at the ridge of the leafy structure (corresponding to the bright fluorescing area in **a**). Red chlorophyll fluorescence also appears under blue light. The photograph was taken four weeks after immature embryo culture. (**f**) Plantlet formation. The roots have developed from somatic embryos. The 'labelled' somatic embryo is located at the bottom of the picture, with its leaf pointing right. The photograph was taken forty days after the initial immature embryo culture.

3.3 Factors that affect the frequency of green shoot regeneration

The plant regeneration frequency from immature embryos of wheat was affected by many factors. On average, 20 plantlets could be regenerated from each immature embryo. If donor plants were grown under the best conditions, the regeneration frequency could reach 60 shoots per immature embryo. Sometimes, over 100 shoots were obtained from a single wheat immature embryo.

3.3.1 Genotype

The frequency of plant regeneration from immature embryo (IE) cultures was genotype-dependent. More than ten varieties of wheat were tested for plant regeneration. Three cultivars, Scamp, Rascal, and Kedong 58, gave higher frequencies of regenerable callus (RC) from immature embryo cultures (**Table 3-1**).

3.3.2 The growth condition of explants

The frequency of plant regeneration is affected by the growth condition of explants. The variation of regeneration frequencies from one genotype in different experiments (**Table 3-1**) indicated that the growth conditions of the donor plants affected the tissue culture response. Immature embryos harvested during winter or early spring usually responded well in tissue culture, thus a higher frequency of plant regeneration could be obtained. When the temperature was high, the explants grew fast and the zygotic embryos developed rapidly (immature embryos could grow to 1-1.5mm in less than 12 days post anthesis). Immature embryos obtained under these conditions usually produced less regenerable callus, and the germination rate of the immature embryos on culture medium was higher.

Table 3-1. Genotype effect on the frequency of formation of regenerable callus from IE cultures.

Exp. No.	Genotype	No. of IE cultured	No. of IE producing regenerable callus	Frequency of RC from IE
1	Scamp	25	22	88%
	K58	44	41	93%
	Rascal	130	70	54%
2	Scamp	98	65	66%
	K58	77	63	82%
	Rascal	262	215	82%
3	Scamp	112	103	92%
	K58	30	30	100%

Note: IE = immature embryo; RC = regenerable callus.

3.3.3 Density effect on tissue culture

When using the same material (i.e. the particular variety harvested on the same day), the plant regeneration frequency was determined by the tissue culture conditions. In one experiment summarized in **Table 3-2**, whole calli derived from twenty-eight immature embryos of Scamp were transferred to five 9cm Petri dishes with regeneration medium.

In **Table 3-2**, Dish 1 to Dish 4 contained four IE derived calli each, and Dish 5 contained twelve calli. Cultures at lower density (Dish 1 to Dish 4) yielded more regenerated plants (on average 7 shoots per IE) than cultures at higher density (Dish 5) (on average 2 shoots per IE). However, if the shoot primordia were counted, there was little difference between the two (average shoots plus primordia per IE, 14 and 12). It seems that in tissue cultures at high density the shoot primordia are hindered from developing into shoots. This was probably due to the limitation of nutrient supply in higher density cultures.

Table 3-2 Density effect on plant regeneration (Scamp).

IE no. per dish	Dish no.	No. of green shoots	No. of shoot primordia	Average no. of shoots per IE	Average no. of shoots + primordia per IE
4	Dish 1	19	40	5	15
	Dish 2	25	30	6	14
	Dish 3	36	35	9	18
	Dish 4	34	10	8	11
	Sub-total	114	115	7	14
12	Dish 5	25	120	2	12

Note: IE = immature embryo.

3.3.4 Dissection and sub-culture of regenerable callus

An increased frequency of plant regeneration could be achieved by dissecting the regenerable calli and separating them on new regeneration medium. In one experiment, all ten cultured immature embryos of Scamp produced regenerable callus (**Table 3-3**). Regenerable calli were separated and transferred onto MS-r medium to regenerate plants. On average, 26 plants were regenerated from each immature embryo.

The potential for plant regeneration may be even higher. Sub-culture of regenerable callus resulted in more green shoot formation. In another experiment, twelve immature embryos were inoculated on MS-b medium. Seven immature embryos, labelled as a, b, c, d, e, f and g, produced regenerable calli (**Table 3-4**). Calli were separated into small pieces and transferred to regeneration medium at a low density (about 6 callus pieces per 9cm dish). When the calli from the first dissection grew big enough, they were separated again and transferred to new regeneration medium (**Table 3-5**). The highest number of regenerated plants was 243 shoots from one embryo. Many of the shoots were formed by micro-tillering. They arose from axillary buds.

Table 3-3 Plant regeneration from ten immature embryos of Scamp after dissection of regenerable calli (results were summarized eight weeks after immature embryo culture).

IE code	No. of callus pieces	No. of shoots on each callus piece	Shoot no. per IE
IE1	7	3, 1, 6, 9, 0, 0, 0	19
IE2	13	3, 6, 1, 2, 7, 2, 8, 9, 0, 0, 0, 0, 0	38
IE3	10	4, 8, 3, 2, 1, 0, 0, 0, 0, 0	18
IE4	7	5, 5, 9, 3, 9, 4, 8	43
IE5	7	1, 2, 3, 1, 3, 5, 11	26
IE6	11	1, 2, 3, 2, 3, 8, 6, 5, 3, 5, 3	41
IE7	7	1, 5, 2, 1, 3, 6, 0	18
IE8	6	2, 4, 3, 8, 9, 0	26
IE9	8	3, 3, 4, 8, 0, 0, 0, 0	18
IE10	8	3, 6, 4, 0, 0, 0, 0, 0	13
Average	8.4		26

Note: IE = immature embryo.

Table 3-4. Green shoot regeneration from immature embryos of Scamp after two sub-cultures (results were summarized ten weeks after tissue culture).

IE code	No. of callus pieces at 1st dissection	No. of shoots arising from each 1st callus piece	Shoot no. per IE
a	14	32,4,28,7,11,29,26,24,27,17,13,13,4,8	243
b	20	(used for photography)	
c	13	1,3,3,5,11,12,5,11,0,0,0,0,0	51 *
d	17	2,7,3,7,2,1,4,3,5,2,7,0,0,0,0,0,0	43 *
e	4	6,4,7,12	29
f	5	25,0,0,0,0	25
g	4	87,1,6,0	94
Average			81

Note: IE = immature embryo. The medians are marked with *.

Table 3-5. Details of subculture of callus from IE a, f and g (underlined in **Table 3-4**).

Callus code	No. of callus pieces at 2nd dissection	No. of shoots arising from each 2nd callus piece	No. of shoots from each 1st callus piece
a-1	12	5,8,4,1,3,11,0,0,0,0,0,0	32
a-2	4	1,3,0,0	4
a-3	10	2,3,6,6,4,2,5,0,0,0	28
a-4	4	2,5,0,0	7
a-5	2	4,7	11
a-6	7	5,5,2,3,3,3,8	29
a-7	6	2,6,3,7,5,3	26
a-8	3	7,11,6	24
a-9	8	3,2,5,4,6,3,4,0	27
a-10	5	3,4,4,4,2	17
a-11	3	4,3,6	13
a-12	5	2,3,3,3,2	13
f-1	6	4,4,5,4,5,3	25
g-1	10	10,12,6,14,15,9,3,7,6,5	87

3.3.5 The effects of osmotic treatment, bombardment and selection

Osmotic treatment

Osmotic treatment reduced the total number of shoots and shoot primordia. **Table 3-6** shows plant regeneration after osmotic treatment of Scamp immature embryo cultures. The three experiments were carried out on the same day, using the same material. Experiment 1 compares the osmotic treatment (20% sucrose) effect on plant regeneration without bombardment (single treatment); Experiments 2 & 3 compare the effect of osmotic treatment (20% sucrose) on plant regeneration under bombardment conditions (double treatments). In Experiment 1 (without bombardment), osmotic treatment greatly reduced the shoot primordium formation (average 5.3 shoot primordia per IE compared with 11.3). In Experiment 2 (under bombardment conditions, with pDB1), osmotic treatment had less effect on shoot primordium formation (average 3.8 shoot primordia per IE compared with 4.3), but green shoot number was lower after osmotic treatment. Controversially, in Experiment 3 (also under bombardment conditions, but with pBECKS.red *(C1/Lc)*), although the number of shoot primordia was much lower after osmotic treatment (average 5.1 shoot primordia per IE compared with 11.2), the green shoot number was higher after osmotic treatment.

Table 3-6 Osmotic treatment and bombardment effects on plant regeneration (in Scamp).

Exp. No.	Osmotic treatment	Plasmid	No. of IE/callus	No. of shoot primordia	No. of green shoots	Average no. of shoots + primordia per IE
1	no	no	32	360	0	11.3
	20% sucrose	no	32	170	0	5.3
2	no	pDB1	32	120	16	4.3
	20% sucrose	pDB1	32	116	7	3.8
3	no	C1/Lc	64	695	19	11.2
	20% sucrose	C1/Lc	64	270	55	5.1

Note: IE = immature embryo.

Bombardment and selection

A set of treatments was designed to test bombardment and selection effects on plant regeneration (**Table 3-7**). Immature embryos were pre-cultured on MS-b for two weeks prior to selection or regeneration. Bombardment reduces green shoot and green leafy structure formation (Treatment III compared with I; IV compared with II). PPT selection could inhibit formation of non-transformed shoots, but it did not totally prevent green leafy structure formation from non-transformed IE (Treatment II). Therefore, a long-time and constant PPT selection was needed. Otherwise these leafy structures could, if the selection pressure was released, develop into shoots.

Table 3-7 Bombardment and selection effects on plant regeneration (in Kedong 58).

Treatment	No. of IE	Bombard-ment	Selec-tion	No. of calli with green leafy structures	No. of calli with shoots	No. of calli with roots
I	48	-	-	48	32	30
II	48	-	PPT5	12	0	0
III	48	pDB1	-	31	14	23
IV	48	pDB1	PPT5	6	0	0

Note: IE = immature embryo.

3.4 Discussion

The application of plant biotechnology to crop improvement will be facilitated by the regeneration of plants from single cells. This ensures that transformants are not chimeric. Plants regenerated from protoplasts are obviously of single cell origin, but the establishment of embryogenic protoplast cultures is highly genotype-dependent and time-consuming, especially for wheat. In contrast, intact plant tissues, such as immature zygotic embryos, can be more efficiently used to regenerate whole plants (Ozias-Akins and Vasil 1982, 1983). Immature zygotic embryos have usually been used as target tissues for transformation of cereals, including rice (Christou *et al.*, 1991), maize (D'Halluin *et al.*, 1992), barley (Wan and Lemaux, 1994), and wheat (Weeks *et al.*, 1993; Vasil *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994). Some reports on maize and wheat transformation have suggested that the transgenic plants originated from single cells because the primary transformants were not chimeric and/or the new gene was inherited in a Mendelian fashion in progeny of transgenic plants (D'Halluin *et al.*, 1992; Becker *et al.*, 1994; Nehra *et al.*, 1994).

To investigate how plants are regenerated from immature embryos, wheat immature embryos were cultured on callus induction medium under continuous observation. One or two days after initial culture, some immature embryos were microprojectile bombarded with reporter genes, so that transformed cells/tissue could be traced. We have found somatic embryos expressing different genes after microprojectile bombardment. They appear as 'red' (expressing anthocyanin regulator genes, **Fig. 3-7b**), 'blue' (expressing GUS activity, **Fig. 3-7f**) and 'green' (expressing green fluorescent protein, **Fig. 3-9a**) somatic embryos. The whole embryos expressed the transgenes with clear cell lineage boundaries. The structures of these embryos are similar to the observations of Ozias-Akins and Vasil (1982) (**Fig. 3-4**), which described as nodular and

globular structures, and notch and leafy structures. Uniformly transgene-expressing somatic embryos provided evidence that single cells in the immature embryos had been transformed and had developed into somatic embryos. It was concluded that somatic embryogenesis in wheat immature embryo cultures occurs from single cells.

The somatic embryos first appeared as nodules. They occurred either independently or clustered and fused together. Most were precocious somatic embryos. They assumed typical zygote-like embryo structures at an early stage. Then their scutella elongated and developed into dark-green leafy structures with many trichomes. Shoot primordia formed quickly and true leaves developed shortly afterwards. Sometimes more than one shoot primordium was found at the base of each green leafy structure. The precocious germination of the embryoids resulted in plant formation. Multiple shoot primordia later developed at the base of each leafy structure. Well-formed zygote-like somatic embryos, though existed, were seldom observed.

It is commonly found that somatic embryos germinate before reaching a mature state. Ozias-Akins and Vasil (1982) believed that the phenomenon of precocious germination is not desirable because it results in a smaller and less vigorous seedling. In this experiment, most shoots were developed from precociously germinating embryoids. They grew fast and they were viable and healthy. Thus precocious germination favoured rapid plant recovery after transformation.

Primordia of axillary buds sometimes formed at the same time as leaf primordia were developing. Therefore, micro-tillering occurred and these structures developed during the germination of the somatic embryos. Not all potential shoot primordia developed into plantlets. Early separation of the somatic embryos increased the frequency of plant regeneration. The regenerated plants were formed from somatic embryogenesis and from micro-tillering.

Chapter Four

WHEAT TRANSFORMATION

4.1 Introduction

The biolistic transformation of wheat was first achieved in 1992 (Vasil *et al.* 1992). Subsequently a number of laboratories have made this procedure routine (Weeks *et al.*, 1993; Vasil *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994). A great deal of effort has been poured into trying to improve the transformation efficiencies by investigation of target tissues, culture media, conditions of growth, selection systems, etc. Ingram *et al.* (2001) has presented a comprehensive review on genetic transformation of wheat.

The identification and selection of genetic transformants require the use of genes that function respectively as reporters of gene expression and selectable markers for recovery of transformants. Reporter genes commonly used in plant research include chloramphenicol acetyltransferase (CAT), β-glucuronidase (GUS) and luciferase (LUC). Although these reporter genes are suitable for quantitative analysis, invasive and ultimately, destructive methods are required for detecting the enzymatic activities of CAT and GUS; hence, they cannot be used to monitor gene expression *in vivo*. The use of GUS is also limited by diffusion of the reaction intermediates. Hence, it is not easy to distinguish gene expression between neighbouring tissues, on the basis of histochemical detection of GUS. NPTII has also been used as a reporter for gene expression (Topfer *et al.*, 1988) but, like GUS, the assays are destructive.

Visual markers such as maize anthocyanin regulatory genes (*C1/Lc*), the firefly luciferase gene (*luc*) and the jellyfish green fluorescent protein gene (*gfp*) are non-destructive reporters and they can be used to monitor gene expression *in vivo* (Chiu *et al.*, 1996; Pang *et al.*, 1996; McCormac *et al.*, 1998a; Vain *et al.*, 1998; Lonsdale *et al.*, 1998a; Lonsdale *et al.*, 1998b; Mudge and Birch, 1998; Baruah Wolff *et al.*, 1999).

In wheat transformation, the most frequently used reporter gene is *gusA* (from *Escherichia coli*, former name *uidA*) (Vasil *et al.*, 1993; Weeks *et al.*, 1993; Nehra *et al.*,

1994; Becker *et al.*, 1994). Recently visual marker genes have also been used in wheat transformation, including the anthocyanin genes *C1, B* and *R* (McCormac *et al.*, 1998a; Chawla *et al.*, 1999; Mentemab *et al.*, 1999); the luciferase gene *luc* (Lonsdale *et al.*, 1998a; Lonsdale *et al.*, 1998b); and the green fluorescent protein gene *gfp* (Pang *et al.*, 1996; Jordan, 2000; Weir *et al.*, 2001; Stewart, 2001). The most commonly used selectable marker gene for wheat transformation is the *bar* gene from *Streptomyces hygroscopicus,* with PPT or bialaphos selection (Vasil *et al.*, 1993; Nehra *et al.* 1994; Becker *et al.*, 1994; Weeks *et al.*, 1993). Other selectable marker genes also used in wheat transformation, including *nptII,* with geneticin (G418) selection (Nehra *et al.* 1994), *CP4* and *GOX* genes, with herbicide glyphosate selection (Zhou *et al.*, 1995).

Agrobacterium-mediated transformation has advantages over microprojectile bombardment, and has become a focus of cereal transformation research in recent years. Attempts to develop *Agrobacterium*-mediated wheat transformation systems have been made by researcher world-wide. The first significant report was by Cheng *et al.* (1997). More recently, *Agrobacterium tumefaciens*-mediated transformation of wheat was achieved by Weir *et al.* (2001) using suspension cultured cells as a model system and green fluorescent protein as a visual marker.

In 1994 when the project was at its early phase, literature on wheat transformation was limited to reports delivery of the reporter/the marker genes *gusA, bar* or *nptII* to wheat immature embryos or regenerable embryogenic callus by microprojectile bombardment (Vasil *et al.* 1992; Weeks *et al.*, 1993; Vasil *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994). In the present study, plasmid constructs with the *gusA* reporter, *bar* selectable marker and two visual markers, the *sgfp (S65T)* gene and the *C1/Lc* genes, have been exploited for wheat transformation. The main DNA delivery method was biolistic bombardment. *Agrobacterium*-mediated wheat transformation was also tested by using the

C1/Lc genes. The IEs/calli bombarded with plasmid pDB1 or pAHC25 (both containing the *bar* gene) were selected on PPT-containing medium. After several rounds of PPT selection on culture medium, the surviving green shoots were transferred to soil and the herbicide Challenge was applied to the leaves to screen for herbicide resistant plants. Putative transgenic plants were analysed by PCR and Southern analysis.

4.2 Transient gene expression by PDS-1000/He

4.2.1 The expression of genes in single cells

Transient gene expression can be detected in the epithelial and sub-epithelial cells of the immature embryo scutella one to two days after gene delivery. Individual green cells, blue cells or red cells can be observed after delivery of the *sgfpS65T* gene, the *gusA* gene or the *C1/Lc* genes into immature embryo scutellum tissue (**Fig. 4-1**).

Scutellum cell division can be found in the middle of **Fig. 4-1b** (red cells). Individual blue cells can be distinguished in **Fig. 4-1c**. Blue colour varies, from a tint to dark blue. In some cells, GUS blue is not present in the nuclei. Various shades of blue indicate the different levels of enzyme activity of cells, probably caused by different copy numbers of the plasmid received by individual cell. Multicellular structures expressing foreign genes could be observed about one week after gene delivery (**Fig. 4-1d**).

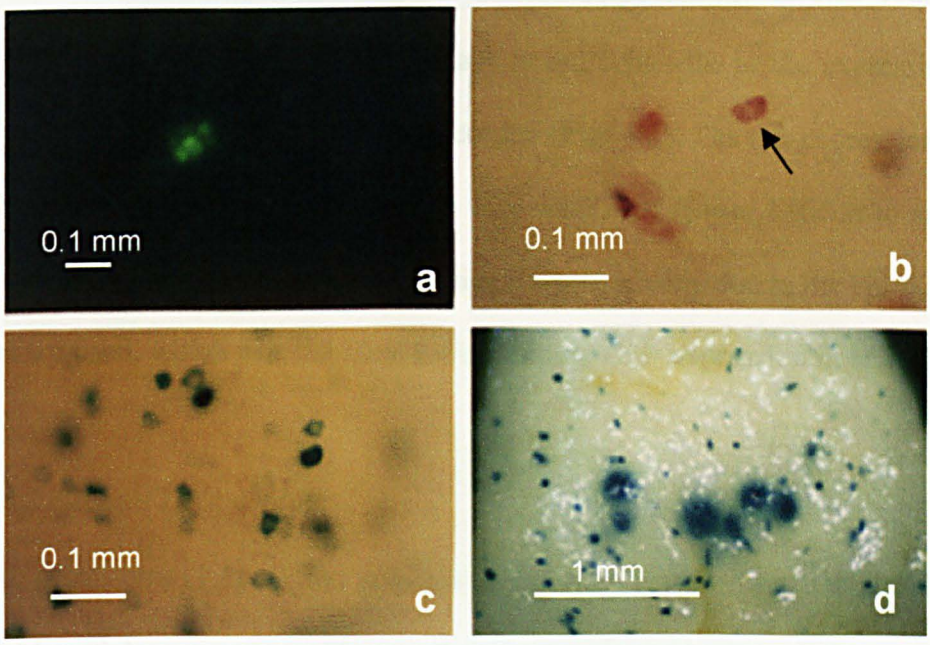


Fig. 4-1 The expression of genes in single cells. **(a)** Transient *sgfp* expression in individual cells of the scutellum. **(b)** Transient *C1/Lc* gene expression, showing cell (in red) division. **(c)** Transient *gusA* expression. Individual blue cells can be distinguished. **(d)** Wheat scutellum (Kedong 58) six days after particle bombardment with plasmid pDB1. Some proliferating callus cells showing GUS staining as a blue area. Single cells with transient GUS staining are still visible.

4.2.2 Factors that affected transient gene expression

Factors that affected transformation efficiency were evaluated on the basis of transient gene expression events. After biolistic bombardment, the number of blue cells (due to *gusA* expression as revealed by the GUS assay) or red cells (due to the expression of the *C1/Lc*) in each scutellum was counted and recorded (**Fig. 4-2**). The *gfp* expression was not assessed, because transient GFP signals were very weak, and it was difficult to examining individual transformed cells. The weak GFP signal was probably due to the epi-fluorescent microscopy we used, which was not ideal for examining GFP expression in callus/tissue.

Particles and plasmid DNA coating

Both tungsten particles (1.1μm) and gold particles (1.0μm) were used for plasmid DNA coating. Gold particles are more uniform and expensive than tungsten particles. In fact, the somatic embryo that expressed GFP (in **Fig. 4-4**) was produced after bombarding with plasmid DNA-coated tungsten particles. An evenly distributed thin layer of DNA on the particles was essential for successful transformation.

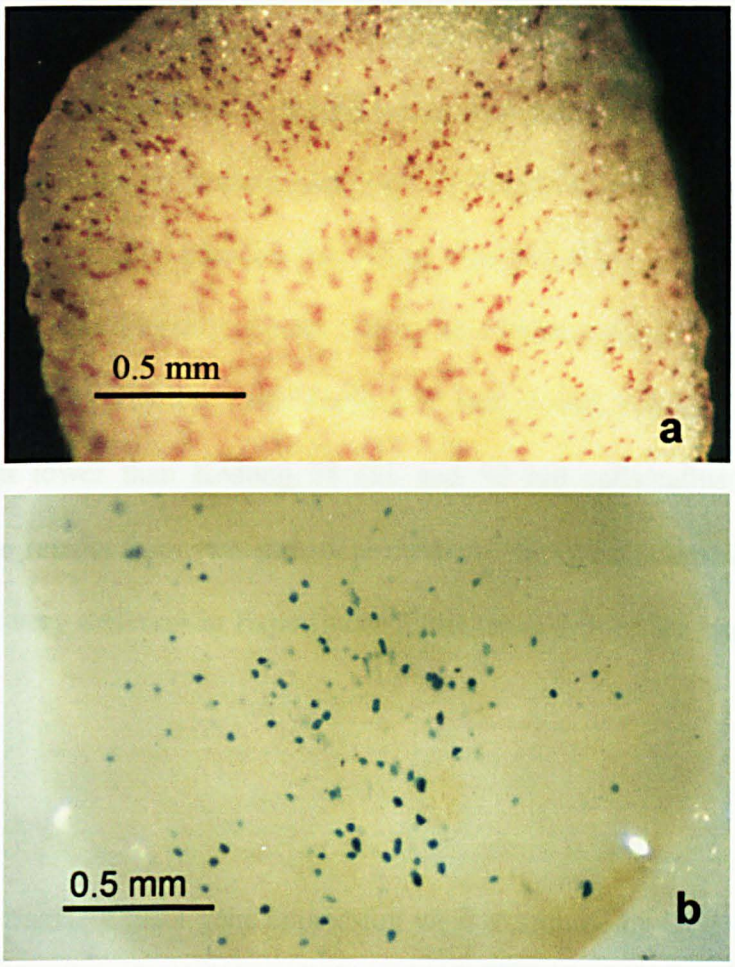


Fig. 4-2 Evaluation of transformation efficiency by counting transformed cells. **(a)** Red cells (transient *C1/Lc* expression). **(b)** Blue cells (transient *gusA* expression after the GUS assay).

Explant genotype

Table 4-1 shows transient *C1/Lc* gene expression determined by counting red cells 24 hours after shooting. In Experiment 1, the materials were bombarded two days after the initial IE culture, and in Experiment 2, the materials were bombarded six weeks after the initial IE culture, there was no osmotic treatment before or after shooting. The plasmid DNA was coated with tungsten particles just before shooting. The number of red cells on each IE/callus varied considerably. Under the same culture conditions, Rascal gave a higher transient gene expression than Tonic (30 and 9 red cells/IE on average in Experiment 1), but lower than Kedong 58 (51 and 92 red cells/callus on average in Experiment 2). The results from two statistical methods, by calculating mean values and using medians, are very different in Experiment 2: the mean values are much higher than the medians.

Plasmid construct

Plasmid effects on transient *gusA* gene expression were examined by GUS assay 48 hours after shooting. The IEs were pre-cultured two days before shooting without osmotic treatment. Two plasmids pAHC25 and pDB1 were tested in three genotypes, Rascal, Scamp and Kedong 58 (**Table 4-2**). The results obtained after calculating mean values and using medians are very close.

In all three genotypes, the transient *gusA* expression of the plasmid pAHC25 was higher than that of pDB1 (**Table 4-2**). In Kedong 58, the difference between pAHC25 (average 41 blue cells per IE) and pDB1 (average 34 blue cells per IE) was not significant. However, in both Rascal and Scamp, the transient *gusA* expression of the plasmid pAHC25 (average 57 and 81 blue cells per IE) was much higher than that of pDB1

(average 9 and 13 blue cells per IE). The poor results in pDB1 was probably caused by plasmid DNA coating quality rather then plasmid DNA itself, because, as can be seen from another experiment shown in **Table 4-4**, the transient *gusA* expression of the plasmid pDB1 was much higher than in this experiment (average blue cells/IE was 37 in Rascal and 31 in Scamp).

Tissue culture conditions

Transient gene expression varied from one experiment to another, even when the same material and plasmid were employed. In this case, the physiological status of the explant played an important role. Freshly isolated and pre-cultured immature embryos responded differently in terms of transient gene expression (**Table 4-3**). It seemed that freshly isolated immature embryos were more susceptible to incorporation of the transgene.

The effect of osmotic treatment

Osmotic treatment before particle bombardment significantly enhanced transient gene expression in the experiments reported by Vain *et al.* (1993). In the experiments described here, sucrose was used as an osmoticum instead of mannitol. Different concentrations of sucrose (5%, 10% and 20%) were added to the callus induction medium to test its effect on transient gene expression. **Table 4-4** shows the effect of the osmotic treatment (20% sucrose in MS-b medium) on transient *gusA* gene expression. The IEs of Rascal and Scamp were pre-cultured for 6 days, and pre-treated on the osmotic medium for 5 hours before particle bombardment. The GUS assay was performed two days later. At this stage, the osmotic-treated IEs were still compact, while the control became watery and soft. In both genotypes, the blue cells of the osmotic-treated IEs were about twice as numerous as

on the control, if the medians are compared; and 3 to 4 times higher if the mean values are compared.

The effects of different osmotic pre-treatments (10% or 20% sucrose in the medium) were compared by determining transient *C1/Lc* expression in Scamp, and the results were listed in **Table 4-5**. The IEs were pre-cultured one day and transferred to osmotic plates 4 hours before particle bombardment. Four days after bombardment, the red cells were counted. The highest transient *C1/Lc* expression came from the 10% sucrose pre-treatment.

The effect of varying duration of the osmotic treatment (10% sucrose in this case) was determined by measuring transient *gusA* expression in Kedong 58 using plasmid pDB1 (**Table 4-6**). IEs were placed on osmotic plates of medium containing 10% sucrose for either 6 hours or 18 hours before particle bombardment. Blue cells were counted two days after bombardment. The results showed no difference of transient *gusA* expression between the two treatments.

Table 4-1 Genotype effect on transient *C1/Lc* expression (red cells were counted 24 hours after bombardment).

Exp.	Geno-type	No. of IE or callus	No. of red cells on each IE or callus	Total no. of red cells	Average cells per IE (callus)
1	Rascal	43 IE	45,28,15,3,26,64,27,37,9,6,12, 20,51,67,64,53,40,32,47,16,2, 5,12,9,9,10,54,2,28,42,40,119, 69,35,19,8,0,2,42,18,44,23,18	1272	30
	Tonic	32 IE	10,6,3,7,6,1,4,7,7,6,8,14, 15,8,3,5,11,8,19,12,4,2,1, 16,20,16,19,13,13,9,9,10	292	9
2	Rascal	14 calli	0,26,1,5,24,25,5,170,182,142, 98,5,15,17	715	51
	K58	10 calli	77,169,23,203,318,42,29,33, 23,0	917	92

Note: IE = immature embryo. The two maximum and minimum values are underlined.

Table 4-2 Effect of plasmid construct on transient gene expression

Geno-type	Plasmid	IE no. for the GUS assay	No. of blue cells on each IE	Total no. of blue cells	Average no. of blue cells per IE
Rascal	pAHC25	6	23, 70, 58, 96, 65, 31	343	57
	pDB1	16	17, 11, 05, 08, 03, 03,16, 28, 04, 07, 02, 05, 11, 07, 06, 15	148	9
Scamp	pAHC25	12	67, 55, 28, 79, 102,144, 35, 34, 66, 70, 151,136	967	81
	pDB1	19	27, 07, 36, 09, 00, 03, 35, 27, 13, 02, 09, 11, 15, 13, 03, 06, 21, 10, 07	254	13
K58	pAHC25	46	52, 60, 22, 70, 53, 61, 48, 54, 32, 62, 09, 36, 27, 45, 50, 84, 34, 14, 12, 81, 29, 28, 59, 25, 48, 12, 64, 75, 45, 14, 22, 47, 46, 22, 04, 16, 46, 00, 11, 04, 65, 50, 95, 54, 55, 31	1873	41
	pDB1	20	26, 82, 15, 53, 50, 44, 38, 09, 25, 66,35, 04, 30, 46, 11, 26, 15, 65, 19, 13	672	34

Note: IE = immature embryo. The two maximum and minimum values are underlined.

Table 4-3 Pre-culture effects on transient gene expression

Geno-type	Pre-culture time	Plasmid	IE no. for the GUS assay	Blue cells (spots) per IE	Average no. of blue cells per IE
K58	0 day	pAHC25	10	167, 120, 30, 77, 190, 132, 31, 199, 116, 59	112
	3 days	pAHC25	10	83, 90, 41, 63, 53, 57, 117, 70, 104, 96	77
	4 days	pAHC25	10	92, 110, 5, 86, 185, 98, 138, 7, 36, 80	84

Note: IE = immature embryo. The maximum and minimum values are underlined.

Table 4-4 Osmotic treatment effect on transient *gusA* expression.

Genotype	Osmotic treatment	Plasmid	IE no. for the GUS assay	Blue cells (spots) per IE	Average no. of blue cells per IE
Rascal	no	pDB1	16	42, 68, 76, 31, 6, 42, 20, 12, 42, 46, 36, 10, 61, 45, 7, 51	37
	20% sucrose	pDB1	14	254, 20, 70, 266, 350, 11, 100, 110, 90, 100, 50, 50, 100, 150	123
Scamp	no	pDB1	16	33, 50, 19, 41, 42, 54, 7, 2, 56, 2, 34, 31, 17, 7, 46, 60	31
	20% sucrose	pDB1	17	153, 6, 71, 88, 5, 360, 97, 400, 8, 10, 80, 1, 115, 9, 110, 300, 210	119

Note: IE = immature embryo. The two maximum and minimum values are underlined.

Table 4-5 The effect of different osmotic pre-treatments on transient *C1/Lc* expression.

Material	Osmotic treatment	IE no. for red cell counting	Red cells (spots) per IE	Average red cells per IE
Scamp	no	25	few, less than 5	<5
	10% sucrose	24	250, 25, 10, 100, 50, 60, 5, 30, 70, 180, 230, 300, 200, 160, 30, 45, 125, 30, 80, 5, 90, 160, 80, 20	97
	20% sucrose	25	40, 60, 20, 50, 80, 300, 50, 50, 200, 300, 20, 10, 50, 50, 10, 20, 30, 5, 15, 5, 40, 50, 60, 40, 30	63

Note: IE = immature embryo. The two maximum and minimum values are underlined.

Table 4-6 The effect of duration of osmotic pre-treatment (10% sucrose)

Geno-type	Osmotic treatment	Plasmid	IE no. for GUS assay	Blue cells (spots) per IE	Average blue cells per IE
K58	6 h	pDB1	20	52, 56, 78, 50, 100, 52, 54, 80, 64, 63, 42, 35, 25, 56, 57, 75, 90, 83, 75, 34	61
	18 h	pDB1	37	29, 40, 135, 25, 96, 140, 22, 19, 11, 4, 20, 34, 76, 60, 120, 65, 75, 55, 73, 60, 44, 55, 40, 105, 16, 100, 67, 55, 37, 100, 30, 60, 30, 100, 65, 65, 120	61

Note: IE = immature embryo. The two maximum and minimum values are underlined.

4.3 Stable gene expression by PDS-1000/He

The expressions of GUS, C1/Lc and GFP have been observed in multicellular structures or well-organised somatic embryos.

4.3.1 The expression of GUS and C1/Lc

Multicellular structures expressing GUS activity can be found in regenerable calli and somatic embryos after the histochemical GUS assay has been performed (**Fig. 4-3**). The dark blue staining in **Fig. 4-3a** indicates strong *gusA* gene expression. A 'blue' somatic embryo reveals GUS activity (**Fig. 4-3b**). Its scutellum has elongated into a leafy structure. Sometimes, GUS activity can be detected in leafy structures (**Fig. 4-3c**). Two patterns have been found: uniform blue (left) or patchy blue (right). Only half of the leafy structure showed GUS activity indicating either that there are fused somatic embryos, or that the structure is chimeric for the transgene. GUS activity can also be detected in the root (**Fig. 4-3d**).

A 'ruby' somatic embryo was observed after the delivery of the plasmid pBECKS.red (containing the *C1/Lc* reporter) into wheat immature embryo scutellum (**Fig. 3-2b**). However, the colour of the embryo changed from red to blue, then grey, and eventually the embryo died. These embryo changes were probable due to internal pH changes associated with cell death.

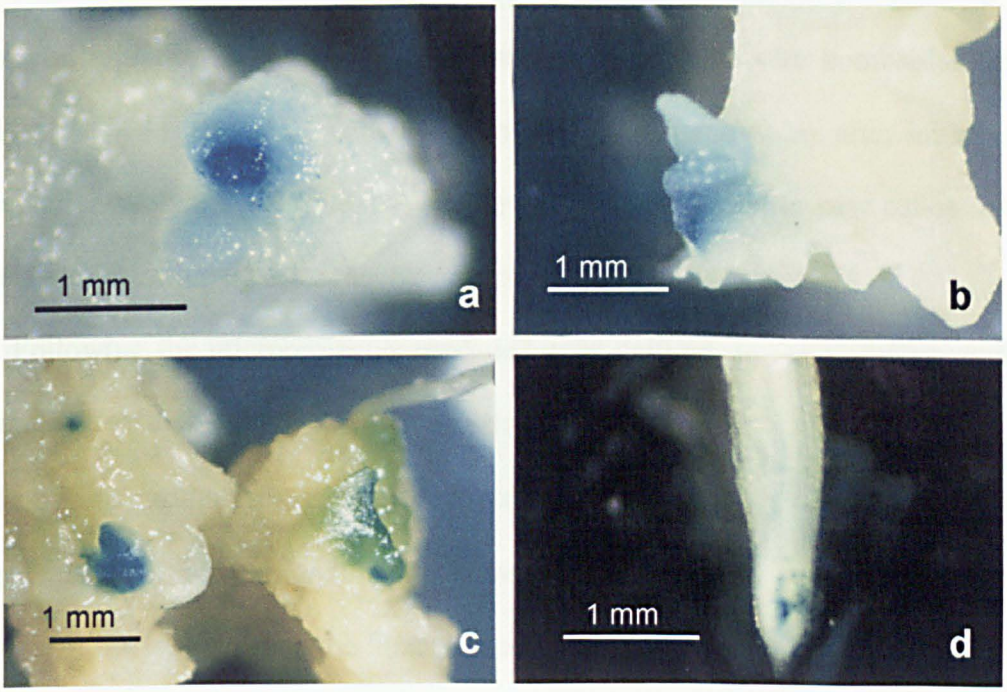


Fig. 4-3 GUS activity in re-differentiated tissues. (**a**) Callus revealing GUS (dark blue) activity. (**b**) A 'blue embryo'. A transformed wheat somatic embryo developed under non-selection conditions. Its scutellum had extended into a leafy structure. (**c**) GUS stains on a leafy structure. Partially transformed somatic embryos from PPT selected embryogenic callus, showing two patterns of chimeric leafy structure. (**d**) GUS activity in a root. A root, from a plantlet which survived several periods of selection, showing GUS activity.

4.3.2 The expression of GFP

Ninety-two freshly isolated wheat (Kedong 58) immature embryos were inoculated in the centre of four 5cm Petri dishes with callus induction medium (MS-h) containing a high sucrose concentration (5% sucrose). The immature embryos were bombarded with the plasmid pBECKS.sgfp(S65T) (coated on tungsten particles) the day after initial culture. One day after shooting, the immature embryos were transferred to new callus induction medium (MS-b) with the normal sucrose concentration (3% sucrose).

Following the microprojectile-mediated gene delivery to the scutellum surface of immature wheat embryos, the cultures were viewed at 1-2 day intervals. Both stereo microscopy and epifluorescence microscopy were used in order to follow the development of regenerating structures and, simultaneously, to screen these for the phenotypic expression of the *gfp* gene *via* the fluorescence of the protein product.

Two days after DNA delivery, early (largely transient) GFP activity was detected within individual cells of the scutellum surface (**Fig. 4-1a**). There was no visible leaching of the GFP fluorescence from the individual expressing cells to neighbouring non-expressing cells.

Pre-embryogenic callus was seen to emerge from the scutella surface within one week from the start of culture; 24 out of a total of 92 immature embryos, which had received DNA, produced such regenerable-type callus. Clusters of calli were separated, if they were not tightly in contact with each other, and individually coded in order to facilitate identification throughout the sequence of observations.

Those multi-cellular structures, in which GFP activity was detected, displayed a variety of patterns with respect to the tissue-types and periods of time for which fluorescence could be observed. Phenotypic *gfp* expression within these independent callus pieces, designated IE1a, IE6a and IE18a, illustrated such diverse patterns (**Fig. 4-4**).

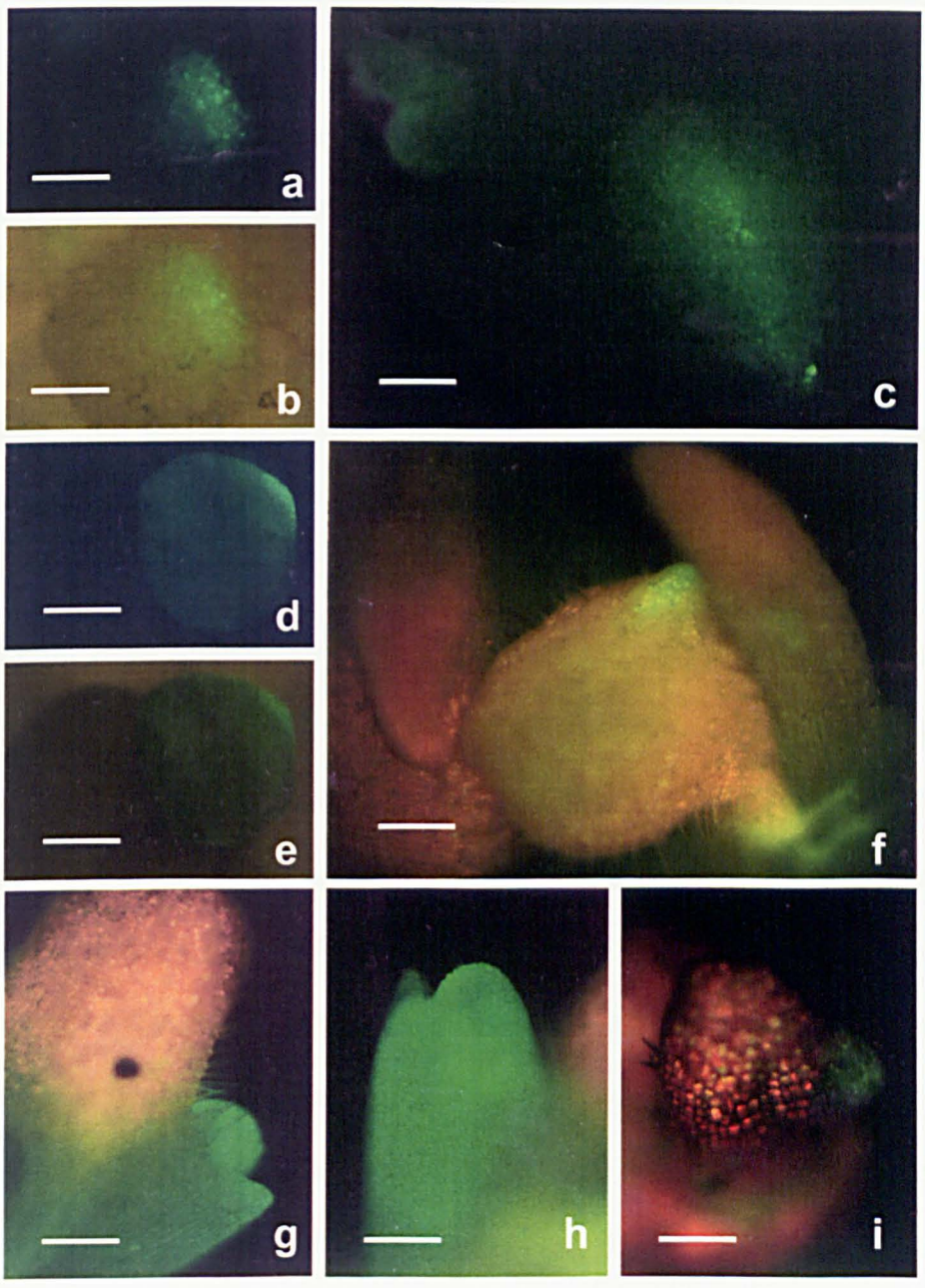


Fig. 4-4 Patterns of *gfp* expression in wheat. **a, b**, and **c** are the same tissue coded IE1a; **d, e** and **f** are the same tissue coded IE6a1 (these 3 images are also used in **Fig. 3-8 a, b & e,** to show the development of this somatic embryo at certain stages); **g, h** and **i** are the same tissue coded IE18a. See next page for details. Bar = 200 μm.

Fig. 4-4 (continued) Wheat immature embryos (Kedong 58) were bombarded with plasmid DNA carrying the *sgfp (S65T)* gene the day after initial culture. The green fluorescence of GFP was traced during callus development using a fluorescence microscope under blue light, with a Nikon B-3A filter set (420-490 nm). (**a**) Callus IE1a emitted green fluorescence at its centre part 12 days after gene delivery. (**b**) As for **a**, with additional normal lighting, showing the whole callus and the location of green fluorescence. (**c**) Callus IE1a four weeks after transformation. The patchy green fluorescence had spread and GFP was also evident on two proliferating cell clusters at the edge. (**d**) Somatic embryo IE6a1 12 days after gene delivery. The whole somatic embryo emitted a mild green fluorescence, with a very bright area at one edge. (**e**) As for **d**, with additional normal lighting. IE6a1 was distinguishable from its neighbour, somatic embryo IE6a2 (right), which did not fluoresce. (**f**) Shootlets from somatic embryos IE6a1 (left) and IE6a2 (right) four weeks after immature embryo culture/gene delivery. GFP remained evident on IE6a1 at the back of the leafy structure (previously a bright fluorescent area). Red chlorophyll fluorescence also appeared under blue light. (**g**) The green fluorescence of GFP emerged at the shoot primordium of IE18a five weeks after gene delivery. The red part was chlorophyll fluorescence on the leafy structure. (**h**) Another view of the same shoot in **g** (from the bottom of the Petri dish). (**i**) Three weeks later than **g**. Green fluorescence remained visible on one leaf after it had grown from the shoot primordium.

Early expression of the sgfp gene

The fluorescent signal in callus IE1a ('IE1' means IE no. 1; 'a' means the first callus) was among the strongest shown by the transformed material recovered in this study, but was unevenly distributed. Twelve days after gene delivery fluorescence was seen, most intensely within a single patch on the surface of the cell cluster (**Fig. 4-4 a & b**). **Figure 4-4 c** shows the same callus two weeks later. The fluorescent area has become enlarged and concentrated in two proliferating cell clusters. The green fluorescence gradually spread over the whole of the tissue. Callus IE1a continued to proliferate and retained areas of green fluorescence for a period of at least 2 months after bombardment. Cells at the surface became visibly enlarged and some areas accumulated chlorophyll, but the callus failed to form primordia. The *gfp* expression in this line was, therefore, limited to undifferentiated cells.

Among calli derived from immature embryo no. 6 (IE6), an organized multi-cellular structure was identified which displayed the *gfp* phenotype. A green-fluorescing somatic embryo, at the globular stage, was identified 12 days after particle bombardment (**Fig. 4-4 d**). The entire area of this somatic embryo fluoresced, albeit to a relatively low level apart from a single intense patch which covered an estimated 2% of the surface area. The green fluorescence, under blue light, of this somatic embryo (IE6a1) made it distinct from an adjacent and otherwise indistinguishable, non-transformed embryo (IE6a2) (**Fig. 4-4 e**). These two globular structures developed leafy outgrowths from which emerged shoot primordia (**Fig. 4-4 f**). In IE6a1, green fluorescence was observed within the early leafy structure, but subsequent development was associated with the accumulation of chlorophyll (seen as red fluorescence when an epifluorescent microscope was used, **Fig. 4-4 f**) and the restriction of GFP fluorescence to an isolated area which corresponded to the

site of maximum intensity in the primary differentiated structure. After six weeks of screening, it was no longer possible to distinguish between IE6a1 and IE6a2 in the basis of fluorescence under blue light. The shoot meristem of IE6a1 did not demonstrate green fluorescence throughout the whole of its development.

Late expression of the sgfp gene

The two patterns of *in vivo* phenotypic activity of GFP, described above, were characterised by a maximal signal soon after DNA delivery. In contrast, the plantlet designated IE18a did not expressing *gfp* until one month after the initial immature embryo culture/gene delivery. The green fluorescence appeared exclusively in an emerging shoot primordium (**Fig. 4-4 g & h**) which was derived from a non-fluorescing callus (IE18a). The IE18a pre-shoot leafy structure also lacked any visible GFP expression. The shoot IE18a continued to show strong fluorescence under blue light for about one week, but subsequent observations revealed a reduction in the visible signal. In the developing plantlet, green fluorescence was restricted to a single nodule in one of the emerging leaf tips (**Fig. 4-4 i**).

In summary, by using *sgfp (S65T)* as an *in vivo* reporter, it was possible to identify multi-cellular structures of wheat that had developed after DNA had been delivered to the early progenitor cell(s). The highest levels of *sgfp (S65T)* expression (as in IE1a) appeared to be associated with the absence of differentiating plant structures. However, in other cases, plantlets could be recovered from green fluorescing somatic embryos and shoot primordium structures.

4.4 Analysis of putative transformants by PDS-1000/He

For various reasons, many putative transformants from earlier experiments were not analysed; some (including the one with 'blue' root) were discarded before transfer into soil. The results reported here were from a few later shooting experiments.

4.4.1 PPT tolerance test and selection

Non-transformed control explants were placed on selection medium for the PPT tolerance test. Immature embryos of Scamp were pre-cultured on callus induction medium MS-b for 1, 2 or 3 weeks before transferring to media PPT0, PPT5, PPT10 or PPT15 (regeneration medium MS-r supplemented with 0, 5, 10 or 15 mg/l PPT, respectively). Twenty immature embryo derived calli were used in each treatment.

Pre-culture one week

No green spots (leafy structures) were visible when transferred to PPT medium. On PPT0 medium, green leafy structures/shoot primordia emerged 5 days after transfer, and developed into shoots and roots. No green shoots developed from calli on PPT5, PPT10 and PPT15, and the calli became brown and died.

Pre-culture two weeks

There were some light green spots (leafy structures) on the calli which were transferred to PPT medium. Green shoots/primordia appeared 2-3 days after transfer to all four media. Most of the green shoots/primordia on PPT5, PPT10 and PPT15 died after 2-3 weeks, and the calli became brown. However, some green leafy structures reappeared during the fourth week of PPT5 treatment. If these leafy structures were transferred to PPT-free medium at this stage, green shoots would develop rapidly and roots would form.

Pre-culture three weeks

Most calli had light green spots/shoot primordia when transferred to PPT medium. Dark green shoots/primordia developed the day after transfer. There was not much difference between the cultures on the four media during the first week after transfer. Two weeks after transfer, roots had developed from the shoots on PPT0. Shoots/primordia on PPT5, PPT10 and PPT15 failed to develop roots, though they remained green for weeks. If the selection pressure was released, roots would develop from these green shoots.

PPT selection is liable to inhibit the formation of shoots. Early selection (before green leafy structure was formed) could avoid some potential "leakage" problems. Medium containing 5mg/l PPT efficiently prevented shoot primordia from developing into shoots. Medium containing 1mg/l PPT was also used for selection.

4.4.2 Herbicide resistance test on leaves

Leaf painting on T_0 plants

T_0 plants bombarded with pDB1 and pAHC25 were screened by leaf painting. These plants had survived several rounds of PPT selection on medium containing 1 to 5mg/l PPT. 1% Challenge solution was applied between two marks (about 5 cm apart) in the middle of green leaves. Three or four days after herbicide painting, the green leaf tissue at the sites of painting became yellow and died. Within one week, the whole painted area, or even the whole leaves became affected. The leaves eventually died.

Most of the leaves died after the development of the symptoms described above which appeared within one week after applying 1% Challenge. However, two leaves survived without developing the symptoms. They belonged to two tillers (S359 and S373) produced after different bombardment events, 85-4 and 85-9. Each of the two tillers set seeds.

Leaf painting on T_1 plants

Two levels of Challenge solution, 0.05% and 0.1%, were tested on T_1 seedlings of lines S359, S360, S361 (from the same cluster of shoots) and S373. 0.1% Challenge was painted in the middle of each L2 leaf, marked with "=" at the edges of the seedlings, and 0.05% Challenge on each L3 leaf, marked with "–". The results of the herbicide test were recorded 10 days after herbicide application (**Table 4-8**). The grades "0", "1", "2" and "3" were used to describe the increasing herbicide effects.

The results in **Table 4-8** do not show clearly which plants are herbicide resistant. In fact, some results look contradictory. It is reasonable to expect that a plant should be more sensitive to higher levels of herbicide than to lower levels. However, there were some plants with unexpected changes after painting with 0.05% and 0.1% Challenge. For example, plantlet S359-1, the L2 leaf was resistant to 0.1% Challenge, but the L3 leaf developed symptoms at a lower level of herbicide (0.05% Challenge). This was probably due to the stage of leaf development. The younger leaf grew more vigorously, so that the cell anabolic activity was high and the cell was more sensitive to herbicide (De Block *et al.*, 1995). **Table 4-9** (a summary of **Table 4-8**) shows that, at the 0.1% Challenge level, about 20% of the seedlings from lines S359 and S361 were resistant to Challenge (with clean leaves), while the control seedlings were sensitive to Challenge by developing varied symptoms.

Table 4-8 Leaf painting on T_1 plants using 0.1% and 0.05% Challenge

Line	Chal-	Seedling number																			
	lenge	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
S359	0.05%	1	0	1	1	2	1	0	0	1	1	3	1	0	2	0	3	1	2		
	0.1%	0	2	0	2	1	3	3	3	3	3	2	2	2	1	0.	2	1	0.		
S360	0.05%	1	0.	0	1	2	2	1													
	0.1%	3	1	3	1	3	3	3													
S361	0.05%	0	0	3	0.	2	1	0	0	2	1	2	2	0	1	1	2	1	1	1	2
	0.1%	0	2	0.	3	3	2	3	1	3	3	3	1	2	3	0.	3	0.	1	3	1
S373	0.05%	0	2	2	2	2	1	0	0	0	2	1	0	0	0	3	2				
	0.1%	3	3	3	3	3	0	3	1	2	1	3	3	1	3	3	3				
CK	0.05%	2	2	1	3	2	1	2	0	2	0										
(K58)	0.1%	3	3	3	3	3	1	2	3	3	3										

Note:

The symptoms are classified from Grade 0 to Grade 3:

Grade 0 or 0.: no visible symptoms, or effects are less than 1/100 of painted area;

Grade 1: less than 1/10 of the painted area with symptoms;

Grade 2: up to half of painted area with symptoms;

Grade 3: more than half of painted area with symptoms, or whole leaf died.

For each seedling, 0.1% Challenge was applied to the L2 leaf; while 0.05% Challenge was to the L3 leaf. The plantlets with unexpected reactions are underlined.

Table 4-9 The number and frequency of seeding with different grades of symptoms in four lines (summary of the results in **Table 4-8**)

Line	Challenge	plant tested	No. of plant (leaf) with different symptoms				Freq. of plant (leaf) with different symptoms (%)			
			G. 0	G. 1	G. 2	G. 3	G. 0	G. 1	G. 2	G. 3
S359	0.05%	18	5	8	3	2	28	44	17	11
	0.1%	18	4	3	6	5	22	17	33	28
S360	0.05%	7	2	3	2	0	29	42	29	0
	0.1%	7	0	2	0	5	0	29	0	71
S361	0.05%	20	6	7	6	1	30	35	30	5
	0.1%	20	4	4	3	9	20	20	15	45
S373	0.05%	16	7	2	6	1	44	12	38	6
	0.1%	16	1	3	1	11	6	19	6	69
CK	0.05%	10	2	2	5	1	20	20	50	10
(K58)	0.1%	10	0	1	1	8	0	10	10	80

4.4.3 Molecular analysis

PCR and Southern blots were carried out to analyse *gfp* expressing and herbicide resistant plants. According to Becker *et al.* (1994), a non-radioactive labelling kit with a DIG labelled probe was used for Southern hybridization. Southern hybridization using a DIG labelled *bar* probe has now been routinely used in wheat in other laboratories (Barro *et al.*, 1998; Cannell *et al.*, 1999).

However, there were some technical problems with my experiments. The sensitivity of the DIG labelled probe was very low. Over 100pg of plasmid DNA was necessary for detection. It was impossible to detect low copy numbers of transgenes in the wheat background. Therefore, no results were obtained from genomic DNA Southern hybridization. Nevertheless, results from PCR and its Southern blot indicated that the transgenes were present in the putative transformants.

4.4.3.1 Analysis of gfp *expression*

Plant IE6a1 (T_0)

PCR was carried out using *gfp* primers to amplify the *gfp* gene in a putative transformed plant. Genomic DNA of IE6a1 (T_0), a putative transformant with the *gfp* gene, together with three controls were amplified with *gfp* primers. The three controls included a positive control (plasmid), a negative control (non-transformed plant genomic DNA), and a blank control (no DNA template). 49μl PCR reaction solution excluding a DNA template was first added to four PCR reaction tubes. Then the DNA templates were added as follows:

Tube 1: 1μl of pBECKS-sgfp (0.1ng/μl)

Tube 2: 1μl of IE6a1 (T_0) genomic DNA (3μg/μl)

Tube 3: 1μl of Kedong 58 genomic DNA (3μg/μl)

Tube 4: 1μl H_2O (as blank control)

PCR programme I was applied for the PCR. After the PCR was completed, 20μl of PCR product from each PCR reaction tube was mixed with 2μl tracking dye and loaded into slots of a mini-gel. Also 1μg λ DNA digested with *Eco*RI and *Hin*dIII was loaded as a size marker. DNA was revealed using an UV trans-illuminator. One clear band, about 700bp, appeared in the positive control (plasmid pBECK-sgfp as template). The exact size of the *gfp* gene amplification using the two primers was 735bp. The blank control was very clean, that meant that there was no contamination of the PCR. However, the fact that there was no band in the PCR products of IE6a1 and Kedong 58, except a weak band near the top of the gel indicated that there were large pieces of genomic DNA template in the samples.

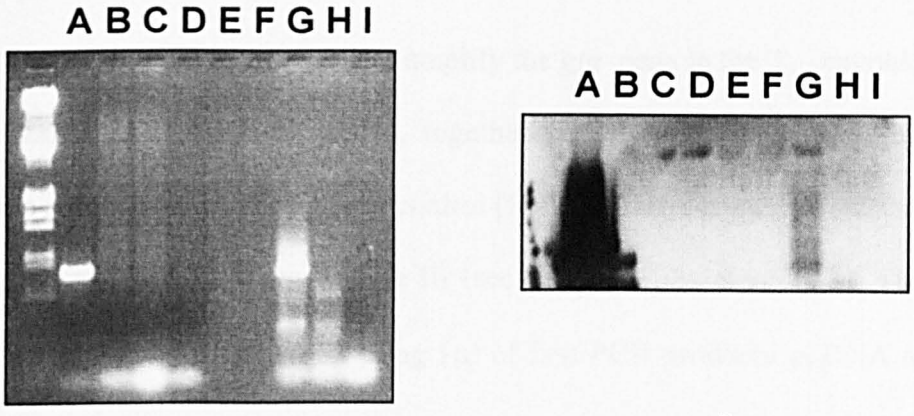
A second round of PCR was performed since no visual band was obtained from the first round of PCR. To four tubes (tubes no. 5 to 8) each containing 49μl PCR reaction solution (excluding the DNA template), the following were added as DNA templates.

Tube 5: 1μl of pBECKS-sgfp (0.1ng/μl) (same as tube 1)
Tube 6: 1μl of PCR product from tube 2 (IE6a1)
Tube 7: 1μl of PCR product from tube 3 (Kedong 58)
Tube 8: 1μl of PCR product from tube 4 (blank control)

PCR programme II was applied for the second round of PCR. After the PCR had finished, 20μl of PCR product from each tube (tube 5 to tube 8) was loaded in a minigel, together with λ *Eco*RI/*Hin*dIII marker. Under UV light, there was one clear band from tube 5 (plasmid as template), plus a band clearly visible from tube 6 (originally IE6a1 genomic DNA as template). This band was the same size as the PCR product from plasmid pBECKS-sgfp. There was no band from the negative control (tube 7) or from the blank control (tube 8).

Another mini-gel was prepared for a Southern blot. 20μl PCR products from tubes 1, 2, 3 and 4 were loaded in Lanes A, B, C and D, while 20μl PCR products from tubes 6, 7 and 8 were loaded in Lanes G, H and I, leaving Lanes E and F blank. The gel was run at 65V for 90 mins, and a photograph was taken under UV light (**Fig. 4-5a**). A clear band appeared in Lane G, that was the second round PCR products using the sample from the first round IE6a1 (T_0) PCR product as a template. The band was at the same molecular weight as the positive control (Lane A, PCR product from plasmid pBECKS-sgfp).

PCR products on the gel (**Fig. 4-5a**, from both the first round and the second round of PCR) were Southern transferred to a Quantum Yield™ Hybridization membrane (for non-radioactive labeling, Promega) and hybridized with a *gfp* probe (details of the procedure were provided in Chapter 2.8). The hybridization signal was visualized on High performance chemiluminescence film (Amersham) by exposing to the hybridized membrane for 10 mins (**Fig4-5b**). Too much amplified *gfp* gene in Lane A resulted in a heavy blot, and the band in Line B (sample from the first round of PCR, using genomic IE6a1 DNA as template) was probably contaminated. A band was formed in Lane G at the same size position as the *gfp* probe. The PCR product of IE6a1 preferentially banded to the *gfp* probe indicating homology (**Fig. 4-5b**, Line G).



a **b**

Fig. 4-5 Amplification by PCR of a 735bp region of the *sgfp (S65T)* gene in genomic DNA from leaves of a T_0 plant IE6a1 (**a**), and Southern blot analysis of products of PCR amplification using DIG labeled *sgfp (S65T)* probe (**b**). DNA samples in lines A, B, C and D are PCR amplification products from the first round of PCR; lines G, H and I are PCR amplification products from the second round of PCR; the far-left line (in **a**) is a λ DNA marker digested with *Eco*RI and *Hin*dIII. DNA templates in each PCR reaction are as following:

Line A	Plasmid pBECKS.sgfp(S65T)
Line B	IE6a1 (T_0) genomic DNA
Line C	Kedong 58 genomic DNA (negative control)
Line D	H_2O (blank control)
Line E	(empty)
Line F	(empty)
Line G	PCR amplification of IE6a1 (from Line B)
Line H	PCR amplification of Kedong 58 (from Line C)
Line I	PCR amplification of H_2O (from Line D)

T_1 plants of IE6a1

PCR was carried out using *gfp* primers to amplify the *gfp* gene in the T_1 generation plants IE6a1. Genomic DNA of 6 T_1 plants, together with a positive control (a plasmid containing the *sgfp* gene) and a negative control (H_2O, no DNA template) were amplified with *sgfp* primers using PCR programme III (see Chapter 2, Section 2.8.3). The second round of PCR was also performed by using 1μl of first PCR products as DNA templates and PCR programme III. After the PCR was completed, 10μl of PCR product from each PCR reaction tube was mixed with 2μl tracking dye and loaded into the slots of a gel. 1μg λ DNA digested with *Eco*RI and *Hin*dIII was also loaded as a size marker. PCR amplifications were visualized in a Bio-Rad Multi-Analyst^{TM}/PC (**Fig. 4-6**).

The exact size of the *sgfp* gene amplification product using the two primers was 735bp. The blank (H_2O) control was very clean, that means there was no contamination of the PCR reaction. A fragment (about 735bp) of the *sgfp* appeared in the positive control (plasmid containing *sgfp* gene as template), while two T_1 plants showed a faint band after the first round of PCR. However, after the second round of PCR, three T_1 plants showed a clear band, at the same position as the plasmid control.

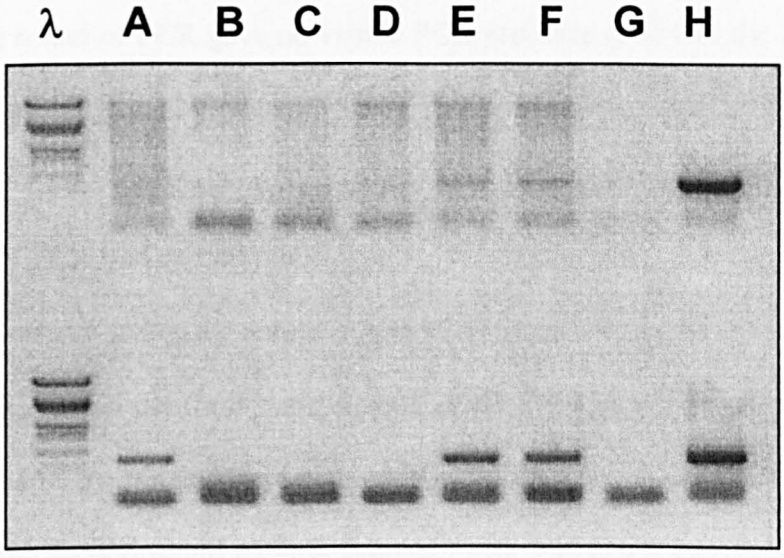


Fig. 4-6 Amplification by PCR of a 735bp region of the *sgfp (S65T)* gene in genomic DNA from the leaves of six T_1 plants of IE6a1. Top row, first round of PCR; bottom row, second round of PCR. The far-left line is λ DNA marker digested with *Eco*RI and *Hin*dIII. DNA templates in each PCR reaction are as following:

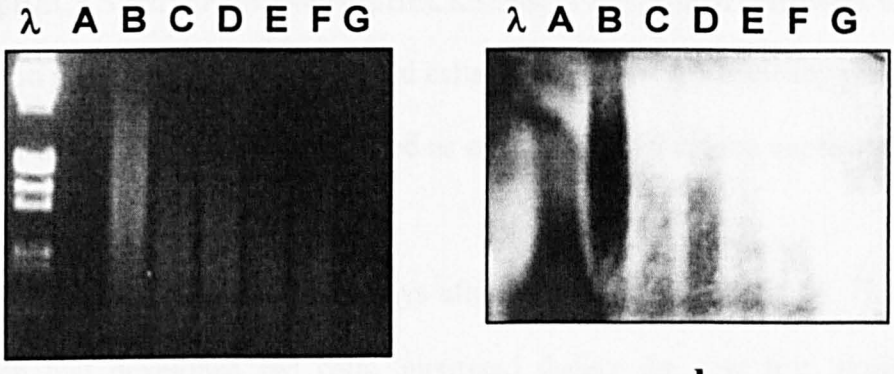
Line A to F	Genomic DNA of six T_1 plants of IE6a1
Line G	H_2O (blank control)
Line H	Plasmid pBECKS.sgfp(S65T)

4.4.3.2 Analysis of herbicide resistant plants

Two T_0 plants/tillers (S359 and S373) were found to be resistant to 1% Challenge applied by leaf painting. The genomic DNA of five of their T_1 plants was used as templates for PCR. The first round of PCR gave no visible PCR products (just like the first round PCR in *gfp* plant) except that the plasmid pDB1 produced a clear band at about the 500bp position (534bp of the *bar* gene product should have been produced by PCR). Therefore a second round of PCR was carried out.

A gel was run using the second round of PCR products and DNA was visualized under UV light. This time, there were smears in all five plant DNA samples, indicating that non-specific PCR products were yielded (**Fig. 4-7a**).

When the second round of PCR products was transferred (on the mini-gel, **Fig 4-7a**) to Quantum Yield™ Hybridization membrane and hybridized with the *bar* probe, bands appeared in all five samples at the same position as the *bar* gene PCR product (**Fig. 4-7b**, Lane B to F, film exposed to the hybridized membrane for 20 mins). PCR amplification from genomic DNA of five T_1 plants preferentially bound to the *bar* probe indicating homology.



a **b**

Fig. 4-7 Amplification by (second round) PCR of a 534bp region of the *bar* gene in genomic DNA from leaves of five T_1 plants which were resistant to the herbicide Challenge (**a**), and Southern blot analysis of the PCR amplification product using a DIG labeled *bar* probe (**b**). DNA samples in each lane are as following:

Far left line: λ DNA digested with *Eco*RI and *Hin*dIII;

Lane A: Plasmid pDB1;

Line B to F: Genomic DNA from the five T_1 plants;

Line G: Blank control (H_2O).

4.5 DNA delivery into wheat cells mediated by *Agrobacterium tumefaciens*

A binary vector, pBECKS.red, was used to test *Agrobacterium*-mediated wheat transformation. It contains the *C1/Lc* reporter complex. Scutellum-derived callus (3 weeks old) of wheat (Kedong 58) was co-cultivated with *A. tumefaciens* strains EHA101(pBECKS.red) or LBA4404 (pBECKS.red). Transformed cells were identified by the change in phenotype (formation of red cells). DNA delivery efficiency was determined by counting red cells, which were formed as a result of *C1/Lc* gene expression (**Table 4-7**).

Red cells could be detected 3 days after co-cultivation (**Fig. 4-8**). The number of calli, which had developed red cells, increased during the first two weeks after co-cultivation. About 60% of the calli expressed *C1/Lc* one week after co-cultivation, and 80% after two weeks. The red cell number in individual calli was also increased. The frequency of red cells was observed to vary for different wheat cultivars and according to callus age, *Agrobacterium* strain and density. High quality explants were important for gene delivery. Callus produced from medium containing acetosyringone (AS) gave a higher frequency of red cell expression. The highest frequency in a single experiment was some 96% based on the numbers of calli targeted, whilst the numbers of red cells in an individual callus varied from 1 to more than 200.

The patterns of transient gene expression are different in *Agrobacterium*-mediated transformation and microprojectile bombardment-mediated transformation. The distribution of red cells from *Agrobacterium*-mediated transformation was not even, but patchy (**Fig. 4-8**). Transformed cells are usually connected to each other, therefore it is difficult to count individual transformed cell. In microprojectile bombardment-mediated transformation, the transformed cells were distributed evenly on the surfaces of the immature embryo scutella or calli (**Fig. 4-2**).

Table 4-7 The efficiency of DNA delivery into wheat (Kedong 58) mediated by *Agrobacterium* strain EHA101(pBECKS.red)

Exp. no.	Total no. of calli	No. of calli with red cells (freq.) after co-cultivation					
		3 days	4 days	6 days	8 days	10 days	16 days
I	19	1	2	4	8 (42%)	10 (53%)	13 (68%)
II	28	7	12	22	23 (82%)	24 (86%)	27 (96%)
III	26	3	11	18	20 (77%)	20 (77%)	21 (81%)
IV	27	0	12	16	17 (63%)	18 (67%)	21 (78%)
Total	100	11	37	60	68 (68%)	72 (72%)	82 (82%)

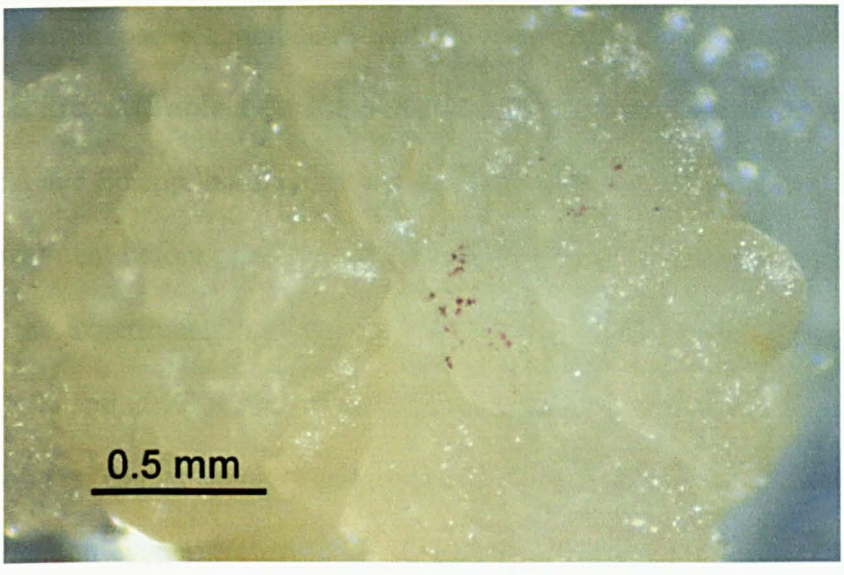


Fig. 4-8 Transient *C1/Lc* gene expression mediated by *A. tumefaciens.* Scutellum-derived callus (3 weeks old) of wheat (shown here from immature embryos of wheat 'Kedong 58') was co-cultivated with EHA101 (pBECKS.red). Photo was taken 3 days after co-cultivation.

4.6 Discussion

4.6.1 Factors that affect transient gene expression

Transient gene expression was affected by several factors, such as density of the plasmid coating on the particles, explant genotype, plasmid construct, pre-culture time and osmotic pre-treatment before particle bombardment. Kedong 58 gives higher Transient gene expression than Rascal (**Table 4-1**). Plasmid pAHC25 seems more effective than plasmid pDB1 in Rascal and Scamp, but there is no significant difference in Kedong 58 (**Table 4-2**). Osmotic treatment before particle bombardment significantly enhanced transient gene expression in the experiments reported by Vain *et al.* (1993). Higher concentrations of sucrose (5%, 10% and 20%) in the callus induction medium also increased transient gene expression. However higher transient gene expression was not necessarily associated with stable transformation events. Nevertheless, it was an indicator of efficient DNA delivery into plant cells. We have recovered putative transformed plants after pre-cultureing immature embryos for one day on callus induction medium containing 5% sucrose before bombardment (section **4.3.2**).

Using the *C1/Lc* red cell marker system as an early indicator, it was possible rapidly to optimise the various parameters affecting DNA delivery, in both a biolistic bombardment delivery system and an *Agrobacterium*-mediated transformation system. However, as *C1* and *Lc* are anthocyanin-biosynthesis regulatory genes, the over expression of the genes is lethal, and may cause cell death. Therefore the *C1/Lc* red cell marker system is not suitable for use as a visual marker for stable transformation.

4.6.2 Efficiency of *bar* selection

The *bar* gene was effective as a selectable marker. However, this approach required a long-term repetitive selection strategy and seemed to suffer from "escapes" from selection

of non-transformed material. Furthermore, choosing the appropriate level of the selective agent with which to challenge the plant tissue is an empirical problem which can be highly case-sensitive. In my experiments, after bombarded with the *bar* gene, the cultures were normally selected on PPT medium for several months, and there were still escapes. Due to technical problems, poor Southern results were obtained, and there is no way to estimate the transformation frequency.

Recently, a new positive selectable marker, *pmi* (*manA* gene from *Escherichia coli* encoding for phosphomannose isomerase) has been applied in maize and wheat transformation (Wright *et al.*, 2001). The transgenic plants were able to metabolise the selection agent, mannose, into a usable source of carbon, fructose. They found that using the selectable marker *pmi* with mannose-containing selection media gave high transformation frequencies and few escapes. They also found that *pmi* performed better than *bar*.

4.6.3 *Agrobacterium*-mediated transformation

In the 1980s, *Agrobacterium tumefaciens*-mediated transformation was limited to dicotyledonous plants and thought to be not readily applicable as a method to produce transgenic cereals (Potrykus 1990), following a number of unsuccessful attempts to transform cereals using *Agrobacterium*. However, the situation has dramatically changed since *A. tumefaciens*-mediated transformation was achieved for rice (Chan *et al.* 1993; Hiei *et al.* 1994) and maize (Gould *et al.* 1991; Ishida *et al.* 1996). Recently, *Agrobacterium*-mediated transformation has been further developed for barley (Tingay *et al.* 1997) and wheat (Cheng *et al.* 1997; Weir *et al.* 2001).

The test of *Agrobacterium*-mediated wheat transformation was carried out in February 1995 using a binary vector, pBECKS.red, which contains the *C1/Lc* reporter complex. Transient C1/Lc expression was observed (**Fig. 4-8**). *Agrobacterium*-mediated

wheat transformation was not my focus, therefore it didn't go further. However, the initial success aided further research in our laboratory and successful barley transformation (Wu *et al.*, 1998).

4.6.4 A new visual marker gene, *gfp*, for wheat transformation

In nature, GFP is made by the jellyfish *Aequorea victoria*, and has been used as a convenient marker in various heterologous systems. The first example of its expression in a graminaceous plant was reported by Sheen *et al.* (1995), where bright GFP expression was detected in maize mesophyll protoplasts. These results have since been extended and applied to other cereals and grasses (Pang *et al.* 1996; Elliott *et al.* 1999; Vain *et al.* 1998; van der Geest and Petolino 1998), including wheat (McCormac *et al.* 1998; Jordan 2000; Weir *et al.* 2001). Jordan (2000) suggested that transgenic wheat plants could be selected on the basis of *gfp* expression alone although the inclusion of antibiotic resistance as a selectable marker could improve the efficiency.

In my study, the experiments involving the *gfp* gene in wheat transformation were tested in June 1996. The *sgfp (S65T)* gene was used, which gives more than 100-fold brighter fluorescent signals than the wild version upon excitation with 490 nm (blue) light, and swifter chromophore formation (Chiu *et al.*, 1996). Putative transformed somatic embryos expressing GFP were observed, and they were selected on the basis of GFP expression only. This was novel at that time. Due to technical problems, there were no convincing Southern results to confirm that the GFP expressing plant was transgenic. However, the results from Jordan (2000), who also used the *sgfp (S65T)* gene for wheat transformation (by particle bombardment), showed that all plants selected on the basis of GFP expression only were later proved transgenic by Southern blots.

A good epi-fluorescent microscope is essential for observing GFP expression. Unlike Jordan (2000) who monitored the GFP using a Leica stereo-fluorescent module

attached to a Leica MS5 stereo microscope, the epi-fluorescent microscope I used was designed for examining thin specimens on slides, not ideal for observing thick tissues/calli on medium. Therefore it was not possible for me to examine the GFP expression of the seeds/seedlings from the GFP expressing plant.

Chapter Five

BARLEY TRANSFORMATION

5.1 Introduction

The research on barley transformation was conducted in the Cereals Research Department, JIC, Norwich. All the methods for barley tissue culture, transformation, selection and molecular analysis are based on the protocols of the Barley transformation Group in the Cereals Research Department, JIC (Harwood *et al.*, 2000).

Lysine is one of ten essential amino acids which are required in animal diets. Dihydrodipicolinate synthase (DHDPS; EC4.2.1.52) (gene *dapA*) catalyses the first reaction of lysine biosynthesis in plants and bacteria. Plant DHDPS enzymes are strongly inhibited by lysine (I-0.5 approximate to 10 mu M), whereas the bacterial enzymes are less (50-fold) or insensitive to lysine inhibition (Vauterin *et al.*, 2000). The transfer of bacteria *dapA* gene into plants has the potential to increase lysine level in plants. For this purpose, a *dapA* gene from *E. coli* was used for barley transformation into barley cv Golden Promise. The construct was prepared in Udine, Italy (during an INCO - COPERNICUS programme funded by the EU). Plasmid DNA was delivered into barley immature embryo scutellum tissue by biolistic bombardment. The *bar* gene, which encodes the enzyme phosphinothricin acetyl transferase (PAT), was used as a selectable marker gene. Co-transformation was carried out by using two plasmids, pDAPH7 and pAL51. The pDAPH7 plasmid contains the *dapA* gene under the control of a 35S promoter; whilst the pAL51 plasmid contains the *luc* gene and the *bar* gene, both under the control of maize ubiquitin (*ubi-1*) promoters. Putative transgenic barley plants were obtained and confirmed by BAR and/or LUC expression. PCR results showed that some plants were DAPA positive. Due to the limited time in JIC, Southern analyses of putative transgenic plants were not completed. Nevertheless the work has been included in this thesis, because another visual marker gene *luc* was involved, and could be compared with the visual marker genes *C1/Lc* and *gfp*, which were used in wheat transformation.

5.2 Results

5.2.1 Bialaphos selection and plant regeneration

The scutellum of the immature embryo (IE) started to grow within a few days of culture initiation. Almost all of the embryos produced callus at the end of the first bialaphos selection (2 weeks after embryo culture). Somatic embryo (SE) structures were observed at this stage. After cutting the callus into small pieces (2 to 4 pieces per embryo) and transferring them to the 2nd selection medium (about 10 IEs per dish), some SEs continued to grow, but most of them de-differenciated and produced new callus (just like the immature embryos on the initial callus induction medium), and secondary somatic embryos. During selection, some callus turned brown and died; some remained compact and grew. Approximately 70% of the IEs survived after the 2nd round of selection. Callus was divided into small pieces and transferred to DBC3B medium (about 5 IEs per dish). About 30% of the IEs survived after the 3rd selection. After transfer of the surviving callus to regeneration medium FWG1B for the 4th and the 5th rounds of selection, some SEs developed green leafy structures, and then leaf primordia emerged, as in the case of wheat somatic embryogenesis. 10% of the IEs produced green shoots (1 to 10 shoots per IE).

After 5 rounds of bialaphos selection (2×BCI5B, DBC3B, 2×FWG1B) (ten weeks), 122 green plantlets (from 77 immature embryos) were recovered and transferred to tubes containing BCI1B medium. However, most of them turned yellow within 2-3 weeks and seemed sensitive to bialaphos. Many callus lines grew readily on FWG1B medium, and expressed luciferase. However they were not regenerable, perhaps due to the prolonged culture period, or over expression of foreign genes. Nevertheless, thirty healthy plantlets (derived from 21 immature embryos) were transferred from the tubes (with BCI1B medium) to soil for further analysis. **Table 5-1** summarised the results of selection.

Transformants were obtained from IEs of all sizes (1.0-1.5mm, 0.5-1.0mm or 0.3-0.5mm) through two experiments (**Table 5-2**). There was no difference in the callus induction frequency between differently sized IEs in the first experiment. In the second experiment, young IEs seemed to achieve a higher frequency of callus induction (96% compared with 87% and 81%) 4 weeks (2×BCI5B) after initial culture. However the difference was reduced two weeks later after DBC3B selection (36% compared with 33% and 40%). In both experiments, the frequency of callus induction from luciferin treated IEs was rather low (64% after 4 weeks selection, and 20% after 6 weeks selection), compared with the figures from the total population (71% and 28% respectively) (**Table 5-2**).

Table 5-1 Summary of bialaphos selection procedure.

Selection	Duration (after inital IE culture)	Selection medium	IE to this medium		Density IE/dish(9cm)	Culture condition
			no.	percentage		
1st	Week 1 and 2	BCI5B	804	100%	20	in dark
2nd	Week 3 and 4	BCI5B	795	99%	~ 10	in dark
3rd	Week 5 and 6	DBC3B	573	71%	~ 5	dim light
4th	Week 7 and 8	FWG1B	222	28%	1 to 5	in light
5th	Week 9 and 10	FWG1B	108	13%	1 to 5	in light
6th	Week 11 to 13	BCI1B	77	10%	1 (in tube)	in light

Note: IE = immature embryo.

Table 5-2 Number and frequency of surviving embryos on each selection medium.

Also showing numbers of herbicide resistant plants.

IE size (mm)	Initial IE no.	IEs which survived after selection				Herbicide resistant no. (freq)	Transgenic plant line code
		BCI5B no. (freq.)	DBC3B no. (freq.)	FWG1B no. (freq.)	BCI1B no. (freq)		
Exp. I							
1.0-1.5	176	98 (56%)	34 (19%)	16 (9%)	7 (4%)	2 (1%)	2A, 7A
0.5-1.0	148	83 (56%)	30 (20%)	6 (4%)	3 (2%)	2 (1%)	21A, 23A
mixed *	42	18 (43%)	6 (14%)				
Subtotal	366	199 (54%)	70 (19%)	22 (6%)	10 (3%)	4 (1%)	
Exp. II							
1.0-1.5	145	117 (81%)	59 (40%)	28 (19%)	7 (5%)	3 (2%)	27A, 28A, 32A
0.5-1.0	180	157 (87%)	59 (33%)	22 (12%)	2 (1%)	1 (0.5%)	88A
0.3-0.5	58	56 (96%)	21 (36%)	5 (8%)	2 (3%)	1 (1.7%)	98A
mixed *	55	44 (80%)	13 (24%)				
Subtotal	438	374 (58%)	152 (35%)	55 (13%)	11 (3%)	5 (1%)	
Total	804	573 (71%)	222 (28%)	77 (10%)	21 (3%)	9 (1%)	

Note: * Used for transient *luc* expression test. IE = immature embryo.

Frequency = IE number after current selection / initial IE number

5.2.2 Transient *luc* gene expression

Transient *luc* gene expression was checked 2-4 days after bombardment. Two to three embryos from each bombardment plate were analysed for *luc* gene expression. For Golden Promise, the frequencies of transient *luc* expression were 81% (34 out of 42 immature embryos) in the first experiment and 100% (55 out of 55 immature embryos) in the second experiment. The transient expression of luciferase in the majority of IEs indicated that plasmid DNA was efficiently delivered into target cells.

5.2.3 Stable transgene expression

Stable *bar* gene expression was tested by painting the leaves with a 1% solution of the herbicide Challenge. Nine lines were herbicide resistant, and six lines expressed the luciferase gene (**Table 5-3**). The expression of the *dapA* gene was not tested.

5.2.4 Polymerase chain reaction (PCR)

Primary PCR results showed that, among the 21 independent lines transferred to soil, 13 lines were *bar* PCR positive. Later analysis results showed that nine lines were herbicide resistant and they were all *bar* PCR positive. Seven of the lines were PCR positive for *luc*, although one line failed to express the luciferase gene. Six of the nine lines were PCR positive for the *dapA* gene. Among these nine transgenic lines, five contained all three genes (HI 2, HI 5, HI 7, HI 8 and HI 9); two contained both *bar* and *luc* (HI 1 and HI 4); one contained both *bar* and *dapA* (HI 6) and one contained *bar* only (HI 3) (**Table 5-3**).

5.2.5 Fertility of transgenic lines

Six of the nine lines were fertile and three were sterile (**Table 5-3**). These three sterile lines contained all three transgenes (*bar*, *luc* and *dapA*). Only two lines (Line HI 8 and Line HI 9) with all three genes were fertile.

Table 5-3 A list of transgenic barley plant lines, showing *bar* and *luc* gene expression results, PCR results and fertility.

Clone No.	Line code	No. of plants	PCR results			Gene expression		Fertility	
			BAR	LUC	DAPA	*bar*	*luc*	fertile	sterile
HI 1	2A	3	+	+		+	+	F	
HI 2	21A	1	+	+	+	+	+		S
HI 3	23A	5	+			+		F	
HI 4	27A	1	+	+		+	+	F	
HI 5	28A	1	+	+	+	+	+		S
HI 6	32A	2	+		+	+		F	
HI 7	88A	1	+	+	+	+	+		S
HI 8	98A	1	+	+	+	+		F	
HI 9	7A	2	+	+	+	+	+	F	
Total	9	17	9	7	6	9	6	6	3

5.3 Discussion

The work using genes *dapA*, *bar* and *luc* for co-transformation in barley was novel at the time when it was carried out in 1998. Although I didn't have time to do Southern analyses of putative transgenic barley plants produced in this experiment, I was involved in molecular analyses of putative transgenic barley plants obtained previously by other members in the same group. Generally speaking, the putative transgenic plants which showed herbicide resistance and were BAR PCR positive could be confirmed by Southern blots (using radio active labelling). The expression of LUC at the plant level (leaves) was also an indication of transformation.

5.3.1 Transformation frequency

In two barley transformation experiments, 804 immature embryos of Golden Promise were bombarded with plasmids pDAPH7 and pAL51. There were a total of nine independently transformed lines that were herbicide resistant and *bar* PCR positive. Some of these lines had more than one primary transformant (up to 5) (**Table 5-3**). For the *bar* gene, the transformation frequency was about 1%. Seven out of the nine lines were PCR positive for the *luc* gene, which was in the same construct as the *bar* gene. Six of the nine lines were PCR positive for the *dapA* gene, which was in a different plasmid construct. The co-transformation frequency for *bar* and *luc* (in the same plasmid construct) was 77%; for *bar* and *dapA* (in a different plasmid construct) was 66%; and for *luc* and *dapA* (again in a different plasmid construct) it was 55%. There may have been some transformants that contained the *luc* and/or the *dapA* gene but did not survive during the bialaphos selection.

5.3.2 Selection efficiency

Bialaphos and PPT are strong inhibitors of glutamine synthetase. It is believed that inhibition of glutamine synthetase blocks the metabolism of ammonia, leading to ammonia

accumulation to levels which are toxic and result in the death of the plants (Tachibana *et al.*, 1986). The *bar* gene, isolated from *Streptomyces hygroscopicus*, encodes the enzyme phosphinothricin acetyltransferase (PAT) (Murakami *et al.*, 1986). The expression of the *bar* gene acetylates the free amino group of PPT and detoxifies it.

The bialaphos system has proved to facilitate very efficient selection of barley transformants which contain the *bar* gene. However, the selection is not tight, therefore there are some escapes. **Table 5-2** shows the efficiency of each selection medium. From a total of 804 immature embryos, about 70% of the IEs (573 IEs) survived after two BCI5B selection steps. Forty percent of the surviving IE lines (222 IE lines) went through DBC3B selection. Half of the lines (108 IE lines) transferred to the first FWG1B medium produced green shoots, and 70% of them (77 lines) survived on the second FWG1B medium. However, only 30% of the lines (21 lines) survived on BCI1B medium. Most plantlets regenerated on the second FWG1B medium were bleached during this last bialaphos selection (on BCI1B medium).

The plantlets with healthy roots on BCI1B medium were not guaranteed to be transgenic. After 6 rounds of bialaphos (concentration from 1 mg/l to 5 mg/l) selection lasting about 3 months, 21 lines survived and were transferred to soil. Only 9 lines proved to be herbicide resistant and *bar* PCR positive. That means about 60% of the surviving lines (after bialaphos selection) were escapes.

The selection pressure of BCI1B medium seems stronger than that of FWG1B, perhaps due to the absence of glutamine in BCI1B. De Block *et al.* (1995) studied the selection mechanism of phosphinothricin *in vitro*. They found that tissues with a high anabolic activity were more sensitive to the toxicity of ammonium than tissues with a low anabolic activity. However, tissues with a low anabolic and high catabolic activity were more sensitive to glutamine deprivation. A reduced glutamine level in the regeneration

medium may help to reduce early escapes, however the plant regeneration frequency will also be affected.

Chapter Six

GENERAL DISCUSSION

6.1 Plant regeneration pathway

Somatic embryogenesis is the development from somatic cells, through an orderly series of characteristic morphological stages, of structures that resemble zygotic embryos (Emons, 1994). The characteristic developmental stages of dicotyledon embryogenesis are the globular, heart and torpedo (=cotyledon) stages. These stages for maize embryogenesis include globular and club-shaped or ovoid stages followed by development of a bipolar embryo axis attached to the scutellum, the embryo's storage organ, the cotyledon-stage. Somatic embryos can develop directly on an explant from organized tissue, from isolated protoplasts or from microspores, or indirectly from callus or cells in suspension culture or primary embryoidic structures (Williams and Maheswaran, 1986). Indirect and direct somatic embryogenesis should be seen as two extremes of one continuum: in indirect somatic embryogenesis the embryos develop up to the (pre)-globular stage, then secondary embryos start to appear from the surface of the primary embryos (Raemakers *et al.*, 1995). In direct somatic embryogenesis, the embryos will not only directly form from somatic cells, they also mature to fully formed embryo proper before secondary embryogenesis occurring under the influence of exogenous hormones (Raemakers *et al.*, 1995).. When embryoids arise from a population of proliferating embryonic cells, such as the typical scutellar callus of cereals, the distinction between 'direct' and 'indirect' somatic embryogenesis becomes trivial, and is perhaps only of interest if the number of mitotic cycles between explant and regenerant is to be minimized, e.g. to avoid somaclonal variation (Williams and Maheswaran, 1986).

Evidence for somatic embryogenesis from the scutellar tissue of immature embryo of wheat was first reported by Ozias-Alins and Vasil (1982). Magnusson and Bornman (1985) provided anatomical observations on somatic embryogenesis from scutellar tissues of immature embryos of wheat. Somatic embryos were observed as early as six days after

culturing immature embryos of wheat *in vitro* on 2,4-D containing nutrient media. Somatic embryos may form from three basic tissue types of scutellum dermal, ground and vascular (Magnusson and Bornman, 1985). However, weather somatic embryos originate from single cells of these tissues or a group of cells is not known in wheat.

During microprojectile bombardment, tungsten and gold particles coated with plasmid DNA can penetrate into or through the dermal cells. Cells being were penetrated by particles and survived the first 48 h after bombardment, have a good chance for foreign DNA to integrate into its genome (Hunold *et al.* 1994). Ideally the transgenic plant is obtained *via* direct somatic embryogenesis from a single transformed cell, or *via* indirect somatic embryogenesis from callus or tissue that originated from a single transformed cell. This is to avoid formation of chimeric transgenic plants.

In my experiments using *sgfp (S65T)* for wheat transformation, immature embryos were pre-cultured for one day before biolistic bombardment. A somatic embryo at about 400 μm expressing GFP was observed 12 days after gene delivery (**Fig. 3-9**). The expression was uniform and had a clear cell lineage associated with the embryo proper. This result indicates that transformation and DNA integration occurred at the single cell stage, which led to formation of transformed somatic embryos. . This result also suggested that somatic embryogenesis process was triggered two days after the initial culture.

Early detection of transformants avoids an unnecessarily long tissue culture and selection period, which not only speeds up the transformation procedures, but also reduces somaclonal variation in the transgenic plants (Bregitzer *et al.*, 1998). The new visual marker gene *sgfp (S65T)* has proved to be a promising marker in wheat transformation (Jordan, 2000). As demonstrated here, GFP can function both as a real-time *in vivo* reporter and as a screenable marker. The major advantage of using *sgfp* as a reporter is

that the whole developmental sequence of transformants can be followed without damaging them.

Pre-culture of wheat immature embryos for 5-7 days before particle bombardment may increase the plant regeneration frequency. However, the chance of getting chimeric plants is also increased. Somatic embryos had been observed as early as six days after subculturing immature embryos of wheat (Magnusson and Bornman, 1985). Microprojectile bombardment performed at this stage results in somatic embryos which are partially transformed. If these partially transformed somatic embryos developed into plants directly, they would be chimeric, as we have seen in many reports. This drawback could be prevented by induction of secondary somatic embryos (Raemakers *et al.*, 1995). Therefore, longer tissue culture periods will be needed, and somaclonal variation in the transgenic plants may be increased.

6.2 Comparison of marker genes in plant transformation

In this investigation, four reporter systems were used for transformation experiments: three in wheat (*gusA*, *C1/Lc* and *sgfp*) and one in barley (*luc*). Both *gusA* and *C1/Lc* were suitable for transient gene expression tests. However, analysis of *gusA* expression was destructive, and it was impossible to follow the dynamic changes of gene expression *in situ* over a period of time. In contrast, the expression of *C1/Lc* was readily followed without any enzyme assay; so the observations were more direct and it was possible to follow dynamic changes of transgene expression *in situ* over a period of time.

By using the *C1/Lc* red cell marker system as an early indicator of transformation, it was possible rapidly to optimise the various parameters affecting DNA delivery, by both the biolistic bombardment system and the *Agrobacterium*-mediated transformation system. However, *C1* and *Lc* are anthocyanin-biosynthesis regulatory genes, whose over

expression is lethal, and may cause cell death. Therefore the *C1/Lc* red cell marker system was not suitable for use in stable transformation.

The signal from another visual marker *luc* is the result of an enzymatic reaction, and the observation of luciferase activity relies on a low light imaging system (luminograph). The expression of LUC from tissues (leaves or roots) of putative transgenic plants can be readily detected. To test transient *luc* expression, it seems impossible to identify individual 'glowing' cells within a target tissue, because a microscope could not be combined with a luminometer (Berthold LUMAT LB9501).

A synthetic green fluorescent protein gene, *sgfp (S65T),* was used during studies of wheat transformation in 1996. The expression of the *sgfp (S65T)* gene in regenerating cultures was followed using epifluorescence microscopy. Green fluorescence was detected within multicellular structures including undifferentiated callus, somatic embryos and shoot primordium structures. The sequential epifluorescent screens allowed detection of the GFP signal within early embryogenic structures without the aid of a selectable marker, thus, this gene can be used as an independent co-transformation marker.

In my experiment, the signal of transient *sgfp (S65T)* expression looked weaker than those from *gusA* and *C1/Lc*. However, the transient *sgfp (S65T)* expression can be efficient (Jordan, 2000), and comparable with the *C1/Lc* system. Both of the systems, *C1/Lc* and *gfp* , are ideal for transient gene expression experiments designed to optimise transformation conditions or to test plasmid constructs.

Although the visual marker genes *gfp* and *luc* have been used in plant transformation, the recovery of the transgenic plants generally relied upon a combination of selectable markers which enabled selection on a herbicide or antibiotic (Pang *et al.*, 1996; Nagatani *et al.*, 1997; Vain *et al.*, 1998; Baruah Wolff *et al.*, 1999). In this study, barley plants with the *luc* gene were obtained by selection *via* the *bar* gene (co-

transformed with *luc*) using bialaphos. The wheat plant with *sgfp* was not selected by use of a herbicide or antibiotic. Instead, it was "picked up" by the signal of GFP activity (**Fig. 3-9, Fig. 4-4**). The *in vivo* detection of the GFP signal was followed in regenerating embryogenic structures of wheat, after gene delivery to immature embryos by microprojectile bombardment. Jordan (2000) confirmed that transgenic wheat plants could be selected on the basis of *gfp* expression alone, although the inclusion of anti-biotic resistance as a selectable marker could improve the efficiency.

6.3 GFP as a promising visual marker for screening and selection of transformants

Assays for most of the reporter genes currently used are destructive; therefore it is not possible to follow the development of a cell produced during an individual transformation event. The major advantage of using *gfp* as a reporter is that the whole developmental process of transgenic material can be followed without damaging the transformants. The *gfp* gene can function as an *in vivo* reporter for co-transformation.

Since the initial optimism about the potential application of *gfp* as a universal reporter, the detection of GFP fluorescence within transformed tissues of various plant species has been associated with highly variable degrees of success. Poor GFP signaling has been attributed to a range of causes including cryptic splicing (Haseloff and Amos, 1995), slow chromophore formation, low fluorescence and inefficient expression (Cubitt *et al.*, 1995). This has indicated that it is necessary to carry out a case-by-case assessment of the validity of applying *gfp* as an *in vivo* marker.

In this study we used a synthetic version of *gfp* which has previously been demonstrated to allow increased efficiency of expression within cells of maize (Chiu *et al.*, 1996). The gene also contained the S65T mutation which confers enhanced fluorescence under blue wavelengths and, thereby, minimizes photobleaching and phototoxicity effects

(Heim *et al.*, 1995); this is clearly beneficial for the sequential screening of material required to undergo differentiation. We have found that, following microprojectile-mediated DNA delivery to immature embryos of wheat, expression of *sgfp (S65T)* produced a detectable and distinctive fluorescence of the intact cells at the scutellar surface. GFP fluorescence could also be detected within the callus outgrowths and in the organized early embryogenic structures and shoot primordia derived from these cells. The GFP marker remained discrete to the expressing cells and organs, and hence allowed unambiguous distinction between neighbouring transformed and non-transformed tissues.

The GFP signal was observed within the transformed callus and differentiating structures over a prolonged period of time (1-2 months) subsequent to the initial DNA delivery, and the cell-discrete signal was located throughout multi-cellular structures arising from (presumably) a single transformed cell, so it seems that these transformation events were stable. The loss of *in vivo* detectability of GFP within the more mature leaf structures may have been due to a change in the efficiency of expression but is more likely to have been a reflection of the complexity of the tissue, including the accumulation of chlorophyllous pigments which can interfere with visualization of the GFP signal. Apart from this observation, it was not possible to relate the intensity of the GFP signal to specific differentiated tissue-types, as there was considerable variation in the fluorescence pattern between the individual somatic embryos.

It is clear from previous reports that GFP detection in intact plant tissues demands a high level of expression of the gene (Baulcombe *et al.*, 1995; Chiu *et al.*, 1996). The CaMV 35S promoter, which was used here to drive expression of the *sgfp (S65T)* gene, is known to confer low/intermediate levels of expression, only, within cells of the cereal species (Last *et al.*, 1991; Christensen and Quail, 1996). Substituting a more efficient promoter may extend the tissue-range of GFP detection. However, both this study and

those of Chiu *et al.* (1996) and Haseloff and Amos (1995) have indicated a possible deleterious effect of very high level *gfp* expression on the differentiation of transformants. Therefore, it may not be advisable to deliberately increase expression. Furthermore it was noted that the demarcation of regenerating plantlets, on the basis of sequential observations of *gfp* expression in early embryogenic structures, provided an adequate screen. By using this strategy, it is not vital that the GFP marker is stably expressed in order for it to function as a transformation marker for co-delivered genes.

The exposure to blue light (in order to detect green fluorescence) seemed not to harm tissue growth and plant development. In this study, the transformed plant IE6a1, which had been repeatedly exposed to blue light, developed normally and set seeds.

It is concluded that *sgfp (S65T)* can function in wheat as a non-destructive co-transformation marker for genes of agronomic relevance. The innocuous nature of this marker, with regard to its release into a field environment, makes it an attractive alternative to herbicide- and antibiotic-resistance markers, but the favoured strategy is likely to involve removal of all marker genes before commercial exploitation of transformed crops.

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